# INVESTIGATION OF RECEPTOR BINDING SITES IN HUMAN TRANSFORMING GROWTH FACTOR ALPHA

By

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## ABBREVIATIONS

| | |
|---|---|
| amp | ampicillin |
| AU | absorption units |
| C | carboxyl |
| Cα | alpha carbon |
| d | deoxy |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetate |
| EGF | Epidermal Growth Factor |
| HCl | hydrochloric acid |
| HPLC | high performance liquid chromatography |
| hTGFα | Human Transforming Growth Factor Alpha |
| IPTG | isopropylthio-β-D-galactopyranoside |
| kDa | kilodalton |
| LB | Luria Bertani Media |
| mg | milligram |
| mL(s) | milliliter(s) |
| mM | millimolar |
| MWCO | molecular weight cut off |
| N | amino |
| NaCl | sodium chloride |
| ng | nanogram |
| nM | nanomolar |
| nm | nanometer |
| OD | optical density |
| PAGE | polyacrylamide gel electrophoresis |
| SDS | sodium dodecyl sulfate |
| TGFα | Transforming Growth Factor Alpha |
| TFA | trifluoroacetic acid |
| Tricine | N-[Tris-hydroxymethyl) Methyl] glycine |
| Tris | tris (hydroxymethyl) aminomethane |
| µg | microgram |
| µM | micromolar |

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

INVESTIGATION OF RECEPTOR BINDING SITES IN HUMAN TRANSFORMING GROWTH FACTOR ALPHA

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Human transforming growth factor alpha (hTGFα) is implicated in the progression of tumor cell vascularization, ultimately leading to metastasis. hTGFα is a member of the epidermal growth factor family, and binds to the EGF-receptor (EGF-R). This research has focused on testing several hypotheses concerning the roles of specific residues and regions of the protein in EGF-R binding and stimulation. Previous work had shown that bacterial expression of hTGFα resulted in the formation of insoluble inclusion bodies, limiting the ability of such systems to yield large amounts of correctly folded hTGFα. Given the technical simplicity of generating recombinant proteins in bacteria, the development of a new expression system for hTGFα in *Escherichia coli* was investigated. The first construct employed the commercial vector, pET22b(+) (Novagen, Madison, WI), in which hTGFα expression is regulated under the tightly

controlled $T_7$ promoter. Recombinant protein is expressed with the *pelB* leader of pectate lyase, allowing efficient secretion of the recombinant growth factor into the periplasm. Expression levels of active hTGFα were lower than expected; therefore, an alternate system was designed. This alternate expression system utilizes a histidine tag, to facilitate purification, together with a thioredoxin fusion partner to express soluble, biochemically active hTGFα.

Our initial mutagenic studies of hTGFα have centered on the effect of mutation in the hinge region connecting the amino and carboxyl terminal domains. Molecular models show the hinge region may have a large impact upon overall hTGFα structure and receptor binding capability. Receptor binding affinities of this hinge residue (Val33) and phenylalanine 15 (Phe15) suggest the presence of a potential EGF receptor binding cavity. This receptor cavity may be a mechanism by which EGF-R elicits a different biological response towards its family of ligands. The Val33 and Phe15 mutants have also shown interesting correlations in receptor binding ability and biological response. Through these mutations, we have demonstrated the importance of both of the N- and C-terminal domains of hTGFα in binding to the EGF receptor.

CHAPTER 1
TRANSFORMING GROWTH FACTOR ALPHA AND ITS INTERACTION WITH THE EPIDERMAL GROWTH FACTOR RECEPTOR

## Introduction

The epidermal growth factor (EGF) family is composed of several protein species containing a common six cysteine motif, $CX_7CX_{4-5}CX_{10-13}CXCX_8C$ (Table 1-1).(1) These cysteines form three disulfide bonds whose proper arrangement is integral for any type of receptor interaction. The first member, for which the family is named, is epidermal growth factor (EGF). Discovered in 1962 by Stanley Cohen, EGF is ubiquitously expressed throughout the body.(2) Other EGF family members include transforming growth factor alpha (TGFα), amphiregulin (AR), cripto, epiregulin and vaccinia growth factor.(3) Our interest lies in one specific EGF family member, transforming growth factor alpha (TGFα), which binds the epidermal growth factor receptor (EGF-R). The purpose of our research is the investigation of putative EGF receptor interactions with specific amino acids in human TGFα. The biological roles of TGFα and EGF-R, described below, provide some insight into the significance of their interaction.

Table 1-1. Sequence Alignment of Epidermal Growth Factor Family Members.

| | |
|---|---|
| **Various members** | |
| Human Amphiregulin | GKNGKNRRNRKKKNPCNAEFQNFCIHG-ECKYIEHLEAVTCKCQQEYFGERCGEKSMKTHSMIDSS |
| Human Cripto | RPRSSQRVPPMGIQHSKELNRTCCLNGGTCMLGS-----FCACPPSFYGRNCEHDVRKENCGSVPH |
| Rat Epiregulin | ----------VQITKCSSDMDGYCLHG-QCIYLVDMREKFCRCEVGYTGLRCEHFFL--------- |
| **EGF** | |
| Human EGF | --------VRNSDSECPLSHDGYCLHDGVCMYIEALDKYACNCVVGYIGERCQYRDLKWWELR--- |
| Mouse EGF | ----------NSYPGCPSSYDGYCLNGGVCMHIESLDSYTCNCVIGYSGDRCQTRDLRWWELR--- |
| Horse EGF | RED-GHYSVRNSYQECSQSYDGYCLHGGKCVYLVQVDTHACNCVVGYVGERCQHQDLR-------- |
| **TGFα** | |
| Human TGFα | --------VVSHFNDCPDSHTQFCFHG-TCRFLVQEDKPACVCHSGYVGARCEHADLLAVVAA--- |
| Mouse TGFα | -DSPVAAAVVSHFNKCPDSHTQYCFHG-TCRFLVQEEKPACVCHSGYVGVRCEHADLLAVVAA--- |
| Rabbit TGFα | --------VVSHFNQCPDSHTQFCFHG-TCRFLVQEDKPACVCHSGYVGARCEHADLLA------- |
| Hamster TGFα | --------VVSHFNECPDSHTQFCFHG-TCRFLVQEEKPACVCHSGYVGARCEHADLLA------- |
| Pig TGFα | --------VVSHFNDCPDSHSQFCFHG-TCRFLVQEDKPACVCHSGYVGARCEHADLLAVVAA--- |
| Macaque monkey TGFα | -DPPVAAAVVSHFNDCPDSHTQFCFHG-TCRFLVQEDRPACVCHSGYVGARCEHADLLAVVAA--- |
| Sheep TGFα | -DPPVAAAVVSHFNDCPDSHTQFCFHG-TCRFLLQEEKPACVCHSGYVGARCEHADLLAVVAA--- |
| Bovine TGFα | --------VVSHFNDCPDSHSQFCFHG-TCRFLVQEEKPACVCHSGYVGARCEHADLLA------- |

## Transforming Growth Factor Alpha

Transforming growth factor alpha, first discovered in 1978, was named sarcoma growth factor for its ability to induce growth in sarcoma growth factors in moloney MuSV-transformed mouse 3T3 cells.(4) Sarcoma growth factor was later renamed TGFα for its ability to transform normal fibroblasts in culture.(5)

TGFα is expressed in the female reproductive system and menstrual cycle, skin, liver, and gastrointestine.(6,7) Secreted mainly during early fetal development and the female reproductive cycle, TGFα does have several interesting responses such as precocious eyelid opening and tooth budding in mice, inhibition of gastric acid secretion and wound healing.(8-10) TGFα also occurs in the largest organ, the skin. When expressed in adult keratinocytes in culture, TGFα shows the ability to autoinduce its expression.(11)

Mann et al. and Luetteke et al. both produced strains of mice containing null mutations in the TGFα gene.(12,13) TGFα -/- homozygous mice possess a phenotype of curly whiskers evident at birth and a pronounced waviness of their coat. These mice have a dramatic rearrangement of hair follicles from that seen in normal mice. Homozygous (-/- TGFα) mice were fertile and their physiological development on the whole was normal. This last result is unexpected since TGFα is known to influence fetal development and mitogenesis. This suggests that some other member of the EGF family substituted in the role of the absent TGFα. The TGFα null mutation is strikingly similar to the mouse

homozygous mutant for the waved-1 (wa-1) gene.(14) In fact, crossbred wa-1 mice and -/- TGFα mice still exhibit the same traits, as would be expected from the cross of two homozygous traits. In essence, the wa-1 mutation and TGFα gene behave like alleles in crossbreeding tests.

Overproduction of the fifty amino acid hTGFα protein can lead to the development of benign and malignant tumors, such as breast, lung, and colon carcinomas.(15-17) hTGFα is also implicated in tumor cell vascularization which is linked to metastatic progression and usually correlates with a poor patient prognosis.(18-20) TGFα, a potent angiogenic factor, stimulates the production of new blood vessel growth *in vivo*.(21) In fact, it is more potent than EGF in producing new capillary growth. Vascularization, or angiogenesis, enables a tumor to increase its number of blood vessels when it reaches a critical cell mass thereby allowing the tumor to receive needed nutrients and oxygen from blood for further growth.(22,23)

One of the biggest areas of research in relation to growth factors is its participation in various types of cancers. Cancer implications of transforming growth factor alpha and the epidermal growth factor family have been known for over 15 years. Because of the structural similarities between growth factors, growth factor receptors and oncogenes, it is possible to extrapolate the potential havoc that could be caused by such agents on the proliferation of cancerous cells. This could happen by a number of mechanisms (note that tumor registries show that approximately 90% of all tumors arise from epithelial

cells). These substrates could serve as a transforming protein, through overexpression, expression at the wrong time or as a mutational alteration of function in the case of the EGF receptor.(24)

TGFα and the closely related epidermal growth factor (EGF) which share roughly 33 percent amino acid sequence identity, both bind to the same EGF receptor (EGF-R). Thus, the EGF-R mediates the biological effects of both EGF and TGFα. To understand the importance of studying EGF receptor interactions with hTGFα, one must begin with understanding the EGF receptor's role in the cell.

## Epidermal Growth Factor Receptor

The EGF receptor, a 170,000 Dalton ubiquitously expressed glycoprotein, consists of three major domains: extracellular, transmembrane and cytoplasmic as seen in Figure 1-1.(1) The extracellular domain, characterized by a high content of cysteines and N-linked oligosaccharides, is the ligand binding domain of the receptor. The transmembrane region of EGF-R, characterized by a span of amino acids that is highly hydrophobic, anchors the receptor in the cellular membrane providing a bridge between the extracellular and cytoplasmic domains. The cytosolic or C-terminal domain consists of several conserved tyrosine amino acids (residues 1068, 1086, 1148 and 1173) adjacent to a tyrosine kinase domain that is common in other proteins having this enzymatic activity.(25) Despite homology to serine kinases, the tyrosine kinase domain has a

strict specificity for tyrosine in proteins autophosphorylated by EGF-R.

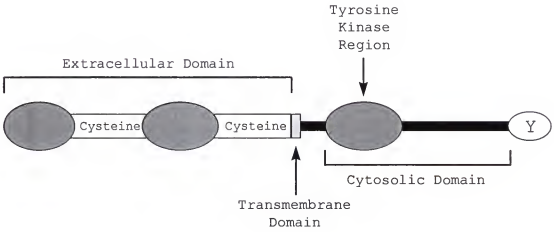



Figure 1-1. EGF Receptor Domains.

EGF-R Tyrosine Kinase Activity

EGF-R produces a biological signaling response through its tyrosine kinase. This signaling process is initiated when a ligand, TGFα, binds the EGF-R extracellular domain. Autophosphorylation of the conserved extracellular tyrosine residues occurs via adenosine tri-phosphate (ATP) as its phosphate source. Several neighboring lysine residues, especially conserved lysine 721, facilitate the transfer of phosphate from ATP to tyrosine. EGF-R tyrosine phosphorylation causes subsequent cellular signaling to occur downstream via second messengers.(26) A variety of proteins have been shown to be phosphorylated after binding of substrate to EGF-R, such as

*ras* oncogene, *erb-B2*, and polyoma middle T antigen. A number of serine kinases are also activated, suggesting a cascade of kinases that is related to growth stimulation.(27)

Ligand binding to the EGF receptor is rapidly followed by internalization. EGF-R/TGFα complexes are either endocytosed in coated pits and degraded by cellular lysozymes or recycled back to the cell surface.(28) The mechanism by which these EGF-R/TGFα complexes are sorted is unknown.

Substrate effects on EGF-R cytoplasmic tyrosine kinase activity is a fundamental question in the study of receptor activation. Ligand induced EGF-R clustering, or dimerization, may provide insight into this phenomena.(29)

EGF Receptor Signaling and Dimerization

EGF receptor kinase activity has been well studied. The signal transfer from the EGF-R extracellular binding domain and the cytosolic tyrosine kinase domain, however, is still under investigation. Schlessinger and Yarden have done extensive studies upon the EGF receptor and have postulated the following two mechanisms for signal transfer, either an intramolecular or intermolecular mode of receptor activation.(30,31) An intramolecular model assumes EGF binding induces a conformational change in the extracellular domain. This change is transmitted by some means through the transmembrane domain to the tyrosine kinase domain which is subsequently activated. On the other hand, an intermolecular model assumes that receptor-receptor interactions are mediated by binding of TGFα, or other ligands,

and this causes activation of the receptor kinase. Schlessinger and Yarden found that EGF induced phosphorylation of its solubilized receptor exhibits a parabolic concentration profile suggesting that a bimolecular step is involved in receptor activation.(30,31) In addition, cross linking of detergent solubilized receptors by different ligands (antibody and lectins) activated the kinase function without the involvement of ligand binding. Lastly, prevention of EGF receptor interactions by immobilization on an inert support abolishes EGF induced activation of the immobile receptor. Thus, this evidence supports the intermolecular model of EGF receptor activation where receptor-receptor interactions are essential for ligand induced activation.

No definitive evidence exists concerning the exact mode of receptor-receptor interaction. Our model of receptor-receptor interaction involves dimerization of monomeric EGF receptors to produce a 2:1 ratio of receptor to ligand as illustrated in Figure 1-2. TGFα would initially bind the EGF receptor. Once bound, TGFα would stimulate a neighboring EGF receptor to interact with the first receptor-ligand complex thereby forming the dimerized structure. In support of this hypothesis, EGF receptors aggregation has been shown to occur subsequent to ligand binding.(32)

## Human Transforming Growth Factor Alpha Structure

Investigation of the unique structural features of hTGFα that initiate binding and activation of the EGF receptor has

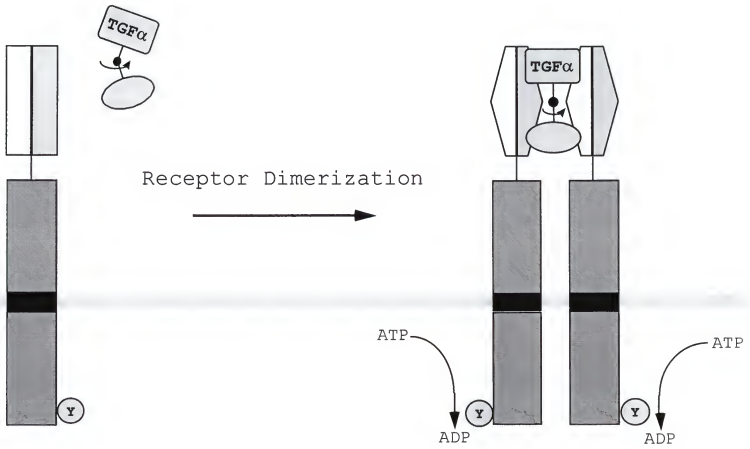



Figure 1-2. EGF Receptor Dimerization Model.

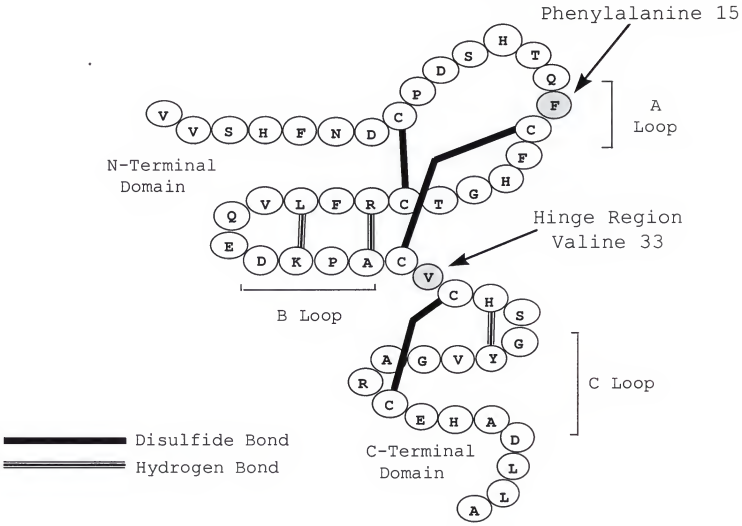



Figure 1-3. Structure of Human TGFα.

been attempted using several methods of structural analysis including X-ray crystallography, NMR and site directed mutagenesis. TGFα's structure is depicted in Figure 1-3.

X-ray Crystallography of hTGFα

Crystallization of hTGFα has been achieved with limited results. Although, the hTGFα/EGF receptor complex was crystallized in 1990 by a German research group, only the crystal lattice groups of ellipsoid type and orthorhombic needles were defined using only the extracellular domain of the EGF receptor. The resolution of X-ray diffraction data was 6 angstroms yielding little specific structural detail.(33) Crystallization of hTGFα was done by Danishefsky et al in 1992 with a resolution of 2.3 angstroms, however no structural detail was included in their paper.(34) No subsequent structural details were published upon either the EGF-R/hTGFα complex or hTGFα alone.

Nuclear Magnetic Resonance Structures of hTGFα

While X-ray crystallography has yielded little structural knowledge of hTGFα, nuclear magnetic resonance (NMR) experiments have produced an enormous amount of information about the solution structure of hTGFα. In 1989, five groups simultaneously published NMR structures for hTGFα.(35-39) These groups found a an antiparallel β-hairpin structure between residues Gly19-Leu24 and Lys29-Cys34 in the N-terminal domain. In addition, another short antiparallel β-sheet was found between C-terminal residues Tyr39-Val39 and His45-Ala46.(40,41) A type II β turn is made by

His35-Tyr38 while a type I hairpin loop is in residues Val25-Asp28. Val1-His4 and Asp48-Ala50 appear to be uninvolved in any regular secondary or tertiary interactions. Harvey et al. suggests His12, Pro30, Phe15, Ala31, Cys8 and Phe23 are in close proximity to one another allowing the formation of a hydrophobic pocket.(42)

Preliminary NMR experiments have also been conducted upon the EGF-R/hTGFα receptor complex by Hoyt et al.(43) This NMR study was performed with the extracellular domain of EGF-R and corroborated several previous mutagenesis studies done on hTGFα. Resonances in the C-terminal domain, especially residues Val39, L48,L49, and A50, of hTGFα, which were flexible in the unbound hTGFα, had a large reduction in mobility upon binding the EGF receptor. Biological studies mutating residues in this region assert the importance of these residues for EGF-R binding.(44-48) Val1 and Val2 maintain their high mobility upon EGF-R binding. Mutagenic studies have shown that truncation of hTGFα residues 1-4 causes no reduction in EGF-R binding capability.

Peptide Mapping and Site Directed Mutagenesis of TGFα

Using recombinant DNA techniques, several research groups have identified functional residues within TGFα that are important for binding and activation of the EGF receptor. Mutation of specific residues in the C-terminal domain and B-loop of hTGFα has resulted in loss of activity.(45,46,48,49) Residues in TGFα's C loop, Leu-48 and Arg-42, have been shown as critical in receptor binding, as have the homologous residues, Leu-47 and

Arg-41, in EGF.(50-54) Synthetic peptides have also been used to locate receptor-binding regions in TGFα, with an almost uniform lack of success. A large series of peptides, which included sequences located in the A-, B-, and C-loops of TGFα, failed to induce DNA synthesis or binding to the EGF receptor.(10,49)

Previous work in our lab using Geysen peptide mapping strategies has helped define possible receptor-binding sites in hTGFα.(55) Sequences of immobilized overlapping hTGFα heptapeptides were assayed binding to the extracellular domain of EGF-R. The results from this assay clearly showed two sequences, corresponding to segments 36-42 and 44-50, that appeared to be bound by EGF-R. These segments contain residues Gly-38, Tyr-39, Gly-40, Arg-42, and Leu-48 which are not only conserved throughout the entire EGF/TGFα family but have also been shown to be critical for biological activity.(56) The use of computer graphics was utilized to visualize the placement of these segments in the three-dimensional solution structure of hTGFα, using data determined by $^{1}H$ NMR techniques(Figures 1-4 to 1-6).(40) Several individual solution phase structures built from NMR measurements adopted a variety of shapes, whose variation could be traced to modified main-chain torsion angles in the hinge residue Val33. All four segments, which significantly bound to EGF-R, were located on a single face of hTGFα. This face formed a continuous surface which stretched from the β-sheet of the B-loop to the flexible C-terminal tail, with sequences corresponding to EGF-R binding peptides defining a distinct cavity. This cavity contained charged residues

predominately on its periphery, while the interior had either hydrophobic or amphiphilic residues. Finally, Phe15 and Val33 lay within this potential EGF-R receptor binding cavity. This type of pattern is common among protein receptor-binding sites as seen in human growth hormone.(57) Receptor-receptor contacts are mediated initially by electrostatic interactions are followed with burial of the hydrophobic core into the receptor.

A notable feature of this model is the use of both the N- and C-terminal domains of hTGFα for binding to EGF-R. These two domains are linked by Val33 which has been shown to be important through site directed mutagenesis studies.(58) Val33 may function as a molecular hinge thereby controlling the relative orientation of the N- and C-terminal domains. Flexibility about this residue has been confirmed through NOE data. Thus, it is possible that the main chain torsion angles of the hinge residue might facilitate the positioning of the two hTGFα domains upon the EGF-R This might be accomplished through alteration of the position of the C-domain relative to the β-sheet of the B-loop in the N-domain. This hypothesis suggests that the flexible C-terminal domain is involved in the initial stages of receptor-ligand interaction. Val33 main chain torsion angles then modify to orient the N-domain onto the EGF receptor thereby inducing a conformational change.

Burial of hydrophobic residues, i.e. phenylalanine 15 (Fl5, N), is driven by regaining the entropy of water.(59) Rearrangement of water molecules around hydrophobic residues disrupts the dynamic network of hydrogen bonding usually present.

Hydrophobic residues do not cause large enthalpy changes, instead these residues increase entropy due to an increase in local order. In addition, F15 lies within the receptor binding cavity suggested by our model. The importance of F15 in hTGFα/EGF-R interactions was investigated in this research.

The results from linear heptapeptides can be questioned as they do not represent hTGFα interactions as a whole. As mentioned previously, other groups have had poor success correlating EGF-R binding and activity with hTGFα peptides, so our results may be under question. DNA recombinant technology was chosen to further investigate this model.

## Specific Aims

Our research upon human transforming growth factor alpha had three specific goals. The initial aim of this research was to develop a protein expression system for the production of correctly folded hTGFα, and site specific mutants using recombinant DNA technology. Using this expression system, we wished to test our EGF receptor dimerization hypothesis by creating mutations in hTGFα.

Our initial target was the hinge residue of hTGFα, valine 33, which lies directly between two cysteines that form two of the disulfide bonds (not to each other). Thus, it is logical to hypothesize that this residue has a large impact upon overall domain orientation, and thus, receptor recognition. Sequence homology alignments suggest that the valine residue is conserved at position 33 for all proteins in the TGFα family. We

hypothesized that residue 33 may be a major determinant for initial EGR receptor binding and have tested this concept through a series of mutations at valine 33.

Our final aim was to determine the importance of residue 15, phenylalanine, in EGF receptor binding and activation. This residue is located in what we believe is the putative receptor binding pocket of hTGFα and also is a highly conserved residue. Phe15 may also have a limited impact upon the EGF receptor dimerization process. This type of hydrophobic core interaction is similar to that proposed for the interactions seen with human growth hormone and its receptor.(57) Again, we wished to test this hypothesis by substitution at position 15.

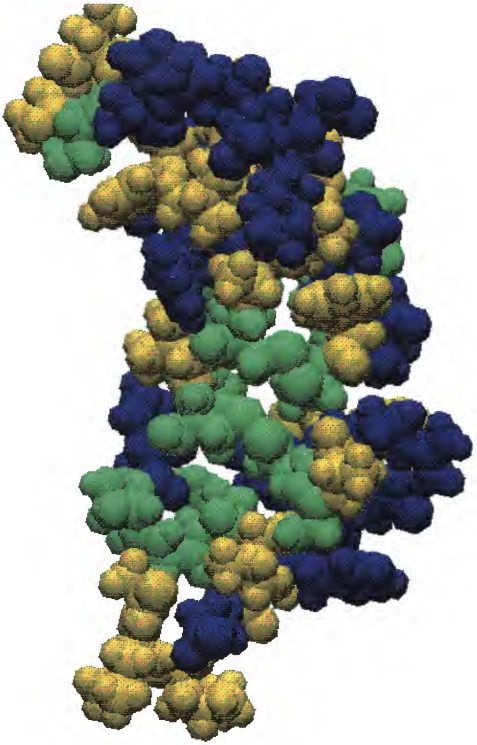


Figure 1-4. Side View of hTGFα. Regions of hTGFα are denoted by the following colors: Blue-Hydrophilic Residues, Green-Amphiphilic Residues, and Yellow-Hydrophobic Residues.

Figure 1-5. Front View of hTGFα. Regions of hTGFα are denoted by the following colors: Blue-Hydrophilic Residues, Green-Amphiphilic Residues, and Yellow-Hydrophobic Residues.

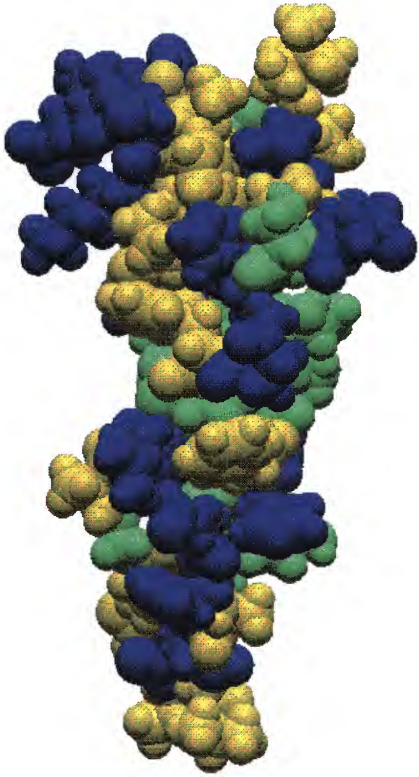


Figure 1-6. Back View of hTGFα. Regions of hTGFα are denoted by the following colors: Blue-Hydrophilic Residues, Green-Amphiphilic Residues, and Yellow-Hydrophobic Residues.

# CHAPTER 2
# EXPERIMENTAL METHODS AND MATERIALS

## Materials

All material sources not specifically listed in the following chapter were of the highest available commercial grade.

### Enzymes

All restriction endonucleases were purchased from New England Biolabs Inc. (Beverly, MA) or Promega (Madison, WI) and used according to manufacturer's specifications.

### Oligonucleotides

All DNA oligomers were synthesized by the Interdisciplinary Center for Biotechnology Research (ICBR) DNA Synthesis Core at the University of Florida (Gainesville, FL).

## Methods

### Sodium Dodecyl Sulfate Polyacrylamide Gels (SDS-PAGE)

All SDS-PAGE gels were produced according to the protocols in Sambrook et al.(60) The protein gels used a Tris-Tricine buffering system to give superior resolution of small molecular weight protein samples.(61)

DNA Preparation

All DNA plasmid preparations were performed according to the established protocols by Sambrook et al.(60) Plasmid preparations were facilitated through the use of the Wizard™ Minipreps DNA Purification System, a commercial kit from Promega (Madison, WI). DNA fragment preparations of all mutant and wildtype hTGFα genes were simplified by the use of the Gel Extraction Kit, a commercial kit from Qiagen Inc. (Chatsworth, CA).

Ligation and Transformation Reactions

Ligation and transformation reactions were done according to published procedures in Sambrook et al.(60) The only deviation from published procedure was done according to the recommended protocol provided by the manufacturer regarding the particular bacterial host strain and commercial source of $T_4$ DNA Ligase.

Bacterial Strains and Vectors

The synthetic hTGFα genes and all mutants were initially ligated to the pCR™ II vector (Invitrogen, San Diego, CA) or the pGEM®-T vector (Promega Corporation, Madison, WI) and transformed into INVαF´ host cells (Invitrogen, San Diego, CA). hTGFα DNA sequences were verified using the Sanger dideoxynucleotide method described below. Initially, recombinant hTGFα was expressed in the bacterial vector pET-22b(+) (Novagen Inc., Madison, WI) as a *pelB* (pectate lyase leader) fusion protein, using *Escherichia coli* (*E. coli*) JM109($DE_3$) cells as the expression host. Final expression of hTGFα was achieved in the pHisTrxpET22b(+) vector

(Protein Expression Core, Gainesville, FL) in *E. coli* BL21($DE_3$) cells.

pHis*Trx*ApET22b(+) [pHisTrx] Vector Assembly

The thioredoxin gene (*trxA*) was obtained by polymerase chain reaction (PCR) from the genomic DNA of *E.coli*.(62-64) The 3' terminus of the *trxA* gene was modified by the addition of a flexible serine-glycine rich linker region. This *trxA* linker was cloned into the *NcoI-SalI* restriction sites in the pET-22b(+) vector. The *pelB* secretion leader was replaced by a tract of ten histidines to facilitate rapid protein purification by metal chelate affinity chromatography.

DNA Sequencing

DNA sequences were determined using the Sequenase kit version 2.0 (United States Biochemical Corp., Cleveland, OH) based upon the Sanger method.(65) The Sequenase kit was used according to the supplier's specifications. DNA sequencing reactions were fractionated on an 8% acrylamide gel containing 40% formamide. Some DNA sequences were determined using the Silver Sequence Kit (Promega, Madison, WI) as per manufacturer's instructions.

Recombinant TGFα Gene Construction

Four deoxyoligonucleotides (NR1, NR3-NR5) of approximately 30 basepairs (bp) each were synthesized with an approximate 15 bp overlap between the neighboring oligomers (Table 2-1).

The oligomers were assembled by overlap extension and amplified using PCR.(66,67) This process generated a synthetic hTGFα gene encoding a 5' enterokinase cleavage recognition site and flanking *EcoR*I and *Hin*DIII restriction endonuclease sites. For each of the substitution mutants of hTGFα, two PCR primers of 23 bp, one primer for each coding DNA strand, were synthesized corresponding to the desired region of substitution in the gene, with the substituted codon flanked by 10 bp of original hTGFα wildtype DNA sequence. These substitution primers, in conjunction with the original hTGFα NR1 and NR5 flanking primers, were used to produce substitutions by using the wildtype hTGFα gene as a template in a PCR reaction. Mutant genes were generated utilizing recombinant PCR in three steps as illustrated in Figure 2-1.(66,67) PCR products were ligated into the pCR™ II vector (Invitrogen, San Diego, CA) and transformed into INVαF' cells. Recombinant native and mutant hTGFα clones were identified initially by restriction analysis. The DNA sequences of the synthetic mutant and native hTGFα genes were determined and the correct nucleotide sequences confirmed. Initially, the *EcoR*I-*Hin*DIII hTGFα DNA fragments were ligated into the pET-22b(+) expression vector (Novagen, Madison, WI), where native and mutant hTGFα proteins were expressed with the *pelB* periplasmic secretion leader sequence. The *EcoR*I-*Hin*DIII hTGFα DNA fragments were ligated into the pHisTrx vector.

Table 2-1. hTGFα DNA Oligonucleotide Primers.

| Oligo Name | Oligo DNA Sequence |
|---|---|
| NR1 | 5'-CGCGAATTCCGCTGACGACGACGATAAAGTTGTTTCTCATTTCAACGACTGCCCGGAC-3' |
| NR3 | 5'-GAACCAGGAATCTGCAGGTTCCATGGAAGCAGAACTGAGTATGAGAGTCCGGGCA-3' |
| NR4 | 5'-GCAGATTCCTGGTTCAGGAAGACAAACCGGCATGCGTTTGCCATTCTGGTTACGTTGGAGCTC-3' |
| NR5 | 5'-GCGAAGCTTATTAAGCCAGCAGATCTGCATGTTCGCAACGAGCTCCAACGTAAC-3' |

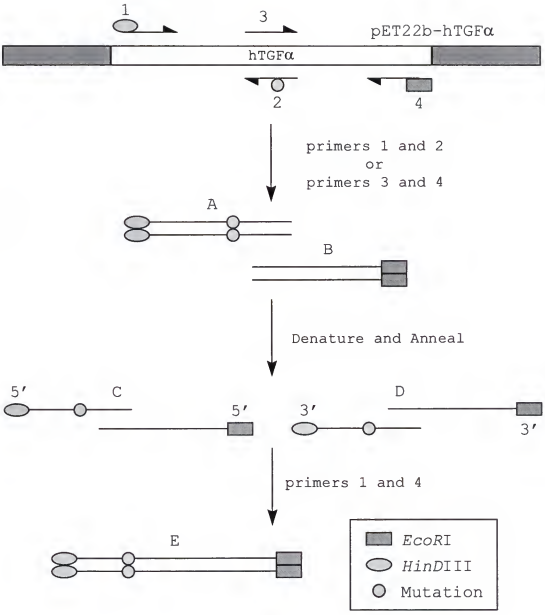



Figure 2-1. Overlap PCR Mutagenesis.

Expression of Recombinant hTGFα Protein in pET-22b(+)

Cell cultures were grown in Luria Bertani (LB) media supplemented with 50 μg/mL ampicillin. At an optical density of 0.7-1.0 (550 nm), hTGFα synthesis was induced with isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration 0.1mM) and grown for an additional 3-4 hours. The cells were collected by centrifugation at 5000 rpm and 4°C using a GSA Rotor in a Sorvall RC2B centrifuge. The cell pellet was resuspended in one tenth the original culture volume of 1M Tris-HCl, 2mM EDTA (pH 9) with 0.1mM dithiothreitol (DTT) and incubated for 30 minutes on ice. hTGFα protein was collected in the supernatant after the cells were pelleted by centrifugation at 10,000 rpm and 4°C using a SS34 rotor in a Sorvall RC2B centrifuge. hTGFα protein was precipitated by the addition of ammonium sulfate to 50% saturation. This slurry was stirred on ice for one hour, after which it was centrifuged at 10000 rpm and 4°C, using a SS34 rotor in a Sorvall RC2B centrifuge, and the supernatant discarded. The ammonium sulfate precipitate was resuspended in one-hundredth the original culture volume in 50mM Tris-HCl (pH 8) with 0.1mM DTT. This solution was then dialyzed into 20mM $NH_4OAc$ (pH 10) using SpectraPor6 (MWCO 1000) regenerated cellulose dialysis membrane. The resulting solution was lypholized and resuspended in one thousandth the original culture volume of ultrapure distilled, deionized $H_2O$ ($ddH_2O$, Millipore).

Expression of Recombinant His*TrxA*-hTGFα Protein

Cell cultures were grown in SOB media (2% (w/v) Bactotryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, pH 7.4) supplemented with 50 μg/mL ampicillin. At an optical density of 0.6-0.8 (600 nm), fusion protein synthesis was induced with IPTG (final concentration 0.4mM) and grown for an additional 3-5 hours. The cells were collected by centrifugation at 5000 rpm and 4°C using a GSA rotor in a Sorvall RC2B centrifuge.

His*TrxA* Fusion Protein Purification

One cell pellet (ca. 166 mL culture volume) was resuspended in 10 mL of 5mM imidazole, 0.5M NaCl, and 20mM Tris-HCl (pH 7.9). Following the addition of phenylmethylsulfonyl fluoride (PMSF, 0.1mM final conc.), the bacterial suspension was immediately sonicated for 5 x 30 sec on power setting 2 using a Virsonic 475 sonicator (Virtis Company Inc, Gardiner, NY) fitted with a micro-probe. The supernatant contained the *TrxA*-hTGFα fusion protein after centrifugation at 24000 rpm (39000 x g) to remove solid cellular debris. This supernatant was filtered through a 0.2μm syringe filter and applied to a charged Poros MC metal chelate affinity column (Perseptive Biosystems, Cambridge, MA). The column was charged with a solution of 0.1M $NiSO_4$ (pH 4.5). *TrxA*-hTGFα fusion protein was applied in its resuspension buffer and eluted with 1M imidazole. Fractions containing *TrxA*-hTGFα fusion protein, identified by SDS-PAGE analysis, were pooled and dialyzed against 50mM Tris-HCl (pH 7.4) overnight at 4°C in SpectraPor6 dialysis membrane (MWCO 1000). Ammonium sulfate was

added to 50% saturation. Fusion protein was pelleted by centrifugation at 10,000 rpm and 4°C, using a SS34 rotor in a Sorvall RC2B centrifuge, then resuspended in $ddH_2O$. This solution was passed through a 0.2μm syringe filter and dialyzed against 50mM Tris-HCl (pH 7.0) to remove any residual salts.

Enterokinase Cleavage of Fusion Protein

Initially, the endoprotease, enterokinase, from Biozyme (San Diego, CA) was used to remove the full length hTGFα protein from its fusion partner, His*TrxA*. However, this enzyme preparation is not highly purified and still contains small amounts of non-specific proteases. Hence, enterokinase from Boehringer Mannheim Corporation (Indianapolis, IN) was used to cleave full length hTGFα protein from its fusion partner, His*TrxA*. The reaction used a 1:20 ratio (w/w; enzyme:fusion protein) in 50mM Tris, pH 7 at 37°C for 9 hours. Ultra-microfilterfuge tubes (MWCO 10,000, Rainin Inc., Woburn, MA) were used to separate uncleaved fusion protein and His*TrxA* fusion partner from the liberated hTGFα protein. The filtrate containing hTGFα protein was lyophilized. The hTGFα protein, dissolved in $ddH_2O$ containing 0.1% trifluoroacetic acid (TFA), was purified using a $C_{18}$ reversed phase column.

Purification of hTGFα

Final purification of native hTGFα was achieved by chromatography on a reversed phase $C_{18}$ column (Model 218TP54, Vydac, Hesperia, CA) using a Rainin HPLC system (Woburn, MA).

Fully active, native hTGFα eluted at 33% acetonitrile (ACN, HPLC grade) using a linear gradient of 10-90% ACN in $H_2O$ (HPLC grade) over 60 minutes (0.1% TFA added to both solvents as an ion pairing agent). Fractions containing the peak at the 33% ACN (20 min) time point were pooled, lyophilized, and resuspended in $ddH_2O$.(68)

hTGFα Antiserum Production

Two milligrams of recombinant hTGFα (Triton Biosciences, Alameda CA) was dissolved in 450 μL of 0.1M $Na_2PO_4$ (pH 7.4). To this solution, 2 mg of keyhole limpet hemacyanin (KLH) and 14 μL of 8% glutaraldehyde solution were added and the resulting mixture was stirred for one hour at 25°C. Thirteen milligrams of glycine were added to quench the reaction. The solution was allowed to stir 10-12 hours to ensure complete quenching. The reaction was diluted by the addition of 500 μL phosphate buffered saline (PBS). Seven hundred and fifty microliters of Freund's complete adjuvant was added to 500 μL of the diluted reaction mixture and mixed with a double headed needle to create an emulsion. A New Zealand albino rabbit was anesthetized with 1 mL of acepromazine maleate (10 mg/mL). After several minutes, the rabbit was injected subcutaneously with equal amounts of the emulsion near each leg. For a basal antigenic level, blood was collected and centrifuged at 14000 rpm (SS34 rotor, Sorvall RC5B centrifuge) for 5 min; the supernatant (serum) was saved. The identical rabbit was boosted three weeks later with the same emulsion. Seven weeks from the original injection date, blood

serum was collected and tested. It showed a high antibody titer (1:1280). The rabbit was exsanguinated and a total of 70 mL blood collected. The resulting serum was lyophilized for long term storage.

Enzyme Linked Immunosorbant Assay of hTGFα Polyclonal Antibody

Enzyme linked immunosorbant assays (ELISA) were performed to establish the hTGFα titer contained in the rabbit serum. Twelve well ELISA plates were coated with 50 μL of a 10 mg/mL solution of recombinant hTGFα in 0.1M $Na_2CO_3$ (pH 9) and left at 4°C for 10-12 hours. After washing the wells with a solution of PBS containing 0.05% Tween™ 20, 50 μL of block solution (PBS, 1 mg/mL bovine serum albumin (BSA), 0.02% $NaN_3$) was added to each well and the plates were maintained at 4°C for one hour. Again, the wells were washed with PBS containing 0.05% Tween™ 20. Serial dilutions of the collected rabbit antisera were made in block solution. Diluted rabbit sera, 100 μL, was added to each well. The negative control was 100 μL of normal rabbit serum. The positive control was 100 μL of polyclonal antibody solution (1:100 dilution, 7/12/92 bleed, G. Schultz, Dept. of OB/GYN, University of Florida). These solutions remained in the wells for one hour at 37°C. After washing, 100 μL of goat anti-rabbit IgG alkaline phosphatase conjugate solution (1:3000 dilution in block solution) was added to each well and incubated at 37°C for one hour. The wells were then washed and developed with 50 μl of Tris-ρ-nitrophenyl phosphate (Tris-NPP) for one hour at 25°C in the dark. The development reaction was stopped with the addition

of 100 µL 3M NaOH in each well. Absorbances were determined at 405 nm using a spectrophotometer (MicroDevices, Vineland, NJ).

Western Immunoblot Analysis of hTGFα Protein

Western immunoblot detection of hTGFα protein followed the procedure of Towbin et al.(69) A rabbit polyclonal antiserum against recombinant hTGFα (some sera was prepared as described above and additional antiserum was a generous gift from Dr. Gregory Schultz, Dept. of OB-GYN, University of Florida) was used as the primary antibody. Goat anti-rabbit IgG antiserum conjugated to alkaline phosphatase (Sigma Immunochemicals, St. Louis, MO) was used as the secondary antibody. Immunoblots were developed with a 1:5 dilution of nitroblue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate solution (NBT, BCIP, Sigma, catalog #B6404, St. Louis, MO) in PBS until the desired intensity was achieved. Development was stopped by washing the immunoblot in $ddH_2O$ for several minutes.

hTGFα Iodination Protocol

Radioactive labeling of hTGFα was accomplished using the procedure of Hunter and Greenwood with the following modifications.(70) A 13 X 100 mm glass tube was siliconized with SigmaCote (Sigma Immumochemicals, St. Louis, MO). After adding a small paper clip as a stirring bar, 5 mg of hTGFα and 20 µL of 250mM $NaH_2PO_4$ (pH 7.4) were added to the glass tube. One milliCurie (mCi) of $^{125}I$-NaI was added to the glass tube and the contents were allowed to stir for 60 seconds at 25°C.

Immediately after 60 seconds had elapsed, 10 µL of chloramine-T (10 mg/mL in 25mM $NaH_2PO_4$, pH 7.4) was added and the reaction mixture was stirred for an additional minute at 25°C. One hundred microliters of $Na_2S_2O_5$ (1 mg/mL in 25mM $NaH_2PO_4$, pH 7.4), 100 µL of 1% KI (25mM $NaH_2PO_4$, pH 7.4), and 100 µL of IgG (1 mg/mL in 25 mM $NaH_2PO_4$, pH 7.4) were added to the reaction tube. The mixture was allowed to stir for several minutes to quench the labeling reaction. The reaction mixture was applied to a G-50 Sephadex (Pharmacia Biotech, Piscataway, NJ) column equilibrated with a running buffer of 10mM HEPES, 150mM NaCl, 1 mg/mL BSA (pH 7.4) and eluted with the same buffer. Fractions were collected at 40 drop intervals with a flow rate of approximately 1 drop per sec. After 40 fractions were collected, 2 µL of each fraction were counted using a Wizard 1470 γ-counter (Wallac Inc., Gaithersburg, MD). Fractions containing the peak activity ($^{125}$I-Labeled hTGFα) were pooled. $^{125}$I-Labeled hTGFα usually elutes in fractions 25-35.

Human Placenta Homogenization

Since human placenta was readily available and cost effective, it was the receptor source utilized in our radioreceptor assays (RRA). Fresh, normal term human placental tissue was obtained from Shands Hospital labor and delivery room. Surface membranes, blood clots, and umbilical cord were removed and discarded. The placenta was then sliced into small segments with a sharp knife. These tissue fragments were washed twice with isotonic buffer containing 50mM Tris, 0.15M NaCl, and 50mM

$CaCl_2$ at pH 7.4. Fragments were homogenized using either a Brinkman Polytron™ homogenizer fitted with the large probe or a Osterizer 14 speed blender. The resulting homogenate was washed with buffer and filtered slowly through 1000 μm nylon mesh. The filtrate was washed again with buffer and progressively filtered through 500 μm then 250 μm nylon mesh. This filtrate was centrifuged at 7500 rpm and 4°C (GSA rotor, Sorvall RC5B centrifuge) for 20 min. The pellet was collected and separated into two fractions, light (membrane tissue) and heavy (red blood cells). Both fractions were resuspended in 15 mL of buffer and homogenized, separately, with a glass DUALL™ tissue grinder (size 23, Kontes Glass Co, Vineland, NJ). The resultant homogenates were frozen at -20°C in 2 mL aliquots. Binding capabilities of the homogenates were ascertained using a RRA. The homogenates had to display a minimum of a 60% ratio between nonspecific and specific binding of hTGFα to be utilized in any RRA.

RadioImmuno- and RadioReceptor- Assays of hTGFα Protein

The buffer used in all radioimmuno (RIA) and radioreceptor assays (RRA) was 50mM Tris, 0.15M NaCl at pH 7.4 containing 1 mg/mL BSA. Samples of 100 μL were incubated with 25 μL of polyclonal antibody (1:50 dilution, produced as described above), raised against recombinant human hTGFα (Triton Biosciences Inc, Alameda, CA), in PBS containing 0.1% BSA for 1 hour at 37°C. $^{125}$I-Labeled hTGFα (~50,000 CPM per 25 μL) was added to each sample and the samples were incubated for an additional hour at 37°C. Immune complexes were subsequently precipitated by the

addition of 100 µL of human immunoglobin G (IgG, 20 mg/mL in PBS) and 4 mL of 15% polyethylene glycol (PEG, MW 8000, 50mM Tris, 0.15M NaCl, pH 7.4) followed by centrifugation at 7000 x *g* for 20 minutes at 4°C. $^{125}$I-hTGFα in the pellet was quantified by Wizard 1470 γ-counter (Wallac Inc, Woburn, MA). A standard curve for hTGFα was generated using serial dilutions of commercial recombinant human hTGFα (Triton Biosciences Inc, Alameda, CA). Displacement data were linearized by the logit transformation.(71) Best fit lines were determined by linear regression analysis using either Microsoft® Excel or Cricket Graph programs. Concentrations of hTGFα in samples were calculated by interpolation from the linear regression curve generated by the hTGFα standard. Radioreceptor assays were performed using the same protocol with the following modifications. One hundred microliters of placental homogenate (1:10 dilution in PBS, 0.1% BSA) was used in place of polyclonal antibody (Ab) and 100 µL of $^{125}$I-hTGFα containing approximately 50,000 CPM was used as the tracer.

Cell Culture

Human corneal fibroblast cells (Dr. Gregory Schultz, OB-GYN, University of Florida) were grown in Trimix media (equal parts of Medium 199 (Sigma), Ham's F-12 Nutrient Medium (Gibco BRL, Gaithersburg, MD) and Dulbecco's Modified Eagle's Medium (D-Mem, Gibco BRL, Gaithersburg, MD)), buffered with 25mM HEPES, pH 7.4, and containing penicillin G (100 Units/mL), streptomycin (100 µg/mL), and ampotercin B (0.25 µg/mL)) supplemented with 10%

(v/v) fetal calf serum (FCS, Sigma Cell Culture Reagents, St. Louis, MO). Cells were passaged by washing with PBS, then harvested with 0.05% (w/v) trypsin, 1mM EDTA (Gibco BRL, Gaithersburg, MD). Cells were pelleted and resuspended in an appropriate volume of TriMix media. Harvested cells were placed in new culture flasks and grown at 37°C.

Methyl-$^3$H-Thymidine Incorporation Assay

Human corneal fibroblast cells were grown to confluency and split into a 24 well plate. The cells were grown at 37°C for 24 hours. Wells were washed three successive times with 1 mL/well of Trimix media containing 1 mg/mL BSA. After the last wash, 1 mL of Trimix media containing 1 mg/mL BSA was added to each well. Fibroblast cells were allowed to grow for 24 hours at 37°C to allow cells to go towards quiescence. After twenty-four hours, the solution was removed from each well and 300 µL of methyl-$^3$H-thymidine (78.5 Ci/mmol, Dupont® NEN Products, Boston, MA, 1 µCi per 300 µL freshly made in Trimix containing 1 mg/mL BSA) followed by 100 µL of sample was added to each well. Cells were incubated at 37°C for up to 24 hours. After the elapsed incubation period, the wells were aspirated of their solutions. A solution of 10% (w/v) trichloroacetic acid (TCA) was added to each well at 1 mL/well and allowed to react for 20-30 minutes. Three washes of methanol (1 mL/well, 200 proof) were performed to remove any remaining acid and cellular debris. The labeled DNA in each well was resuspended in 1 mL of 1N NaOH. Samples (900 mL sample dissolved into 4 mL of scintillation fluid (Scintisafe

Plus™ 50%, Fisher Chemical Company, Fair Lawn, NJ)) were counted using a Beckman LS 5000CE liquid scintillation counter (Fullerton, CA).

Protein Concentration Determination

Protein concentrations were determined using the BioRAD Protein Assay kit (BioRAD, Hercules, CA). Serial dilutions of bovine plasma gamma globulin (BioRAD Standard I) were used as a protein standard. As noted, certain protein concentrations were determined by measuring the optical density of the sample measured at 280 nm. Using a calculated extinction coefficient of 15700 $M^{-1}$ $cm^{-1}$ for His*TrxA*-hTGFα proteins, concentrations were obtained that were in excellent agreement with the BioRAD assay.

CHAPTER 3
PROTEIN EXPRESSION SYSTEMS

## Introduction

Human transforming growth factor alpha (hTGFα) has been studied for over 20 years and several research groups have produced recombinant hTGFα using a variety of strategies. The production of active hTGFα protein is complicated by the requirement for fully oxidized cysteines in three disulfide bonds (Figure 3-1). Any hTGFα protein without the requisite three disulfide bonds will not bind to the EGF receptor.(72) Thus, research groups sought a system capable of producing native hTGFα containing the correct disulfide pairings with varying successes.(45,49) Three main methods of producing hTGFα are eukaryotic expression, prokaryotic expression and chemical synthesis.

## Eukaryotic Expression Systems

Investigators have chosen eukaryotic expression systems mainly for producing post-translational modifications of expressed proteins. This includes glycosylation, a modification that is seen on the pre-pro form of hTGFα.(73,74)

Eukaryotic expression systems utilize two main methods for introduction of foreign genes, shuttle and retroviral vectors.

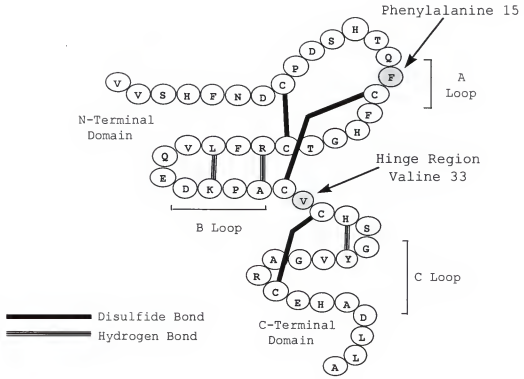



Figure 3-1. Structure of Human Transforming Growth Factor α.

Shuttle vectors which can be either viral or yeast in nature are hybrids of animal and bacterial plasmids.(75) These vectors can be manipulated to handle foreign DNA and replicate in both bacterial and animal cells. The initial phases of cloning a foreign gene can be done in a bacterial host, while foreign protein expression proceeds best in the animal cell host.(75)

Retroviral transfection is often stable, however the use of shuttle vectors can produce either stable or transiently expressed recombinant genes. The establishment of stable transfected cells occurs at least a magnitude less frequently than does the establishment of a transiently expressed cell line. Transient gene expression is amenable to short term experiments,

however, due to cell lysis or failure to stably integrate, recombinant gene expression slowly declines over time.(75) Techniques used to manipulate retroviral shuttle vectors are also similar to that of *E. coli* plasmids, yet, there are major differences that make this type of foreign gene expression more laborious. Shuttle vector transfection of a cell line can take several weeks to months to occur through drug selection protocol. If high titer recombinant retroviruses are used, infection of virtually all individual cells in a cell population can be achieved.

One of the first harvested sources of hTGFα occurred in the culture medium of human melanoma A2058 cells.(76) However, this human cell line produced only 1 μg hTGFα per liter of cell culture medium. Massague purified hTGFα from the same transformed cell line for a yield of 160 micrograms per liter of cell culture.(77) This success was achieved through the use of large cell culture rolling bottles. Although this is an impressive feat, the time and resources used to produce only microgram amounts of hTGFα are impractical for most applications.

A more recent example of hTGFα expression involves the use of retroviral vectors. Pogrebnoi et al. were able to infect human epidermoid carcinoma A431 cells with a retroviral vector containing the hTGFα gene.(78) This stable, transfected cell line secreted 30-35 ng hTGFα per million cells. This cell line is useful for *in vivo* experiments involving hTGFα interactions but expression levels are still too low to use A431 cells as a source of hTGFα.

The convenience of yeast expression systems has improved. This trend is produced in part by the fact that the manipulation of yeast shuttle vectors is similar to that of standard bacterial plasmid techniques. Yeast hosts, such as *Sacchromyces cerevisiae* and *Pichia pastoris*, also create post-translational modifications of expressed proteins through N- and O-linked glycosylation and amidation. The possibility exists for the production of expressed protein with unequal levels of post translational protein modifications. In addition, yeast expression systems often produce the correct protein folding and disulfide bonds formation, as seen with hTGFα. One of the most successful systems to date for expression of hTGFα utilizes the eukaryotic host *Sacchromyces cerevisiae* (*S. cerevisiae*).(45) In this expression system, the yeast peptide mating pheromone alpha factor protein is attached to the amino terminus of hTGFα. Alpha factor peptides are efficiently secreted into the medium by yeast with the α mating type.(79,80) Thus, alpha factor facilitates secretion of alpha factor-hTGFα fusion protein into the growth media. This system is quite prolific, producing approximately a milligram of hTGFα per liter of culture.

Prokaryotic Expression Systems

Most efforts at hTGFα expression have employed the prokaryote, *Escherichia coli* (*E. coli*).(47,49,81) Since bacterial expression hosts have been used with success on many eukaryotic proteins.

*E. coli* possess two cell membranes, the outer membrane and the cytoplasmic membrane, which are separated by the periplasmic space. Proteins located in the periplasm or the outer membrane are synthesized in the cytoplasm and exported through the cytoplasmic membrane. These proteins are always secreted due to N-terminal leader sequences which target the export of any protein attached to it. Very few *E. coli* proteins are naturally secreted into the extracellular growth medium.(82) Thus, the purification of secreted foreign proteins in the growth medium is simplified by the reduced number of indigenous *E.coli* proteins.

One feature that must be discussed is the export of hTGFα into the periplasm which contains a relatively low concentration of reducing species the *E. coli* cell. If the hTGFα protein was produced in the highly reducing environment of the cytoplasm, few, if any disulfides would be present in the protein.(83) Previous investigators have had to refold their expressed hTGFα proteins to yield an active protein.(49,81) Active hTGFα means it contains all of the correct disulfide pairings, i.e. the correct isomer.

## Chemical Synthesis of hTGFα

The chemical synthesis of small peptides is a well established technique. The synthesis of peptides of 50 residues or less using $N^{\alpha}$-9-fluorenylmethyl-oxycarbonyl (Fmoc) or $N^{\alpha}$-tert-butyloxycarbonyl (Boc) chemistry is highly efficient.(84) Chemical synthesis also allows the incorporation of non-naturally

occurring amino acids and for chemical modification of amino acid side chains. For structure function analysis, the use of novel amino acid structures allows a more thorough probing of interactions than afforded by the 20 naturally occurring amino acids.(85) However, problems still result from undesirable side reactions during synthesis, cleavage and deprotection chemistry.(84)

Several researchers were able to chemically synthesize hTGFα using solid phase techniques.(86,87) As seen with *E.coli* and yeast expression systems, synthetic hTGFα must be refolded to produce an active protein as synthesis only produces incorrectly paired disulfides.

Initially, researchers thought that merely the C loop of hTGFα was required for EGF receptor activation. This is not the case as shown by Schultz, Defeo-Jones, and Tam who have synthesized portions of hTGFα which have shown no significant biological response.(49,88,89)

## Preliminary hTGFα Protein Expression Efforts

### Expression Vector Selection

We chose to explore an *E. coli* expression system as it has been technically well characterized and easy to manipulate. We would also avoid post-translational modification of hTGFα, for example glycosylation such as those seen in yeast systems. The lack of uniformity of post-translational modifications makes it difficult to characterize the extent and type of glycosylation.

Initial attempts to clone hTGFα were performed in the pINIII*OmpA* expression vector.(90,91) This vector system contains an *OmpA* secretion leader, from the outer membrane protein A, which facilitates export of proteins into the periplasm.(92) The pINIII*OmpA* vector had been used by a previous group with mixed results.(47) Unfortunately, the vector we obtained had a defective multiple cloning region. Thus, expression of hTGFα protein was not detectable.

Subsequently, we chose to evaluate the commercial vector, pET-22b(+), from Novagen, Inc. (Madison, WI). This vector contained several desirable features, including a *pelB* leader, $T_7$ RNA polymerase transcription promoter and IPTG/*lac* induction.

The *pelB* secretory leader originates from the enzyme pectate lyase.(93-95) It facilitates the export of proteins into the periplasmic compartment of the cell. This is advantageous since the periplasm has a lower reducing potential as compared to the cytoplasm. This loss of reductive capability aids the formation of disulfide pairings in hTGFα (Figure 3-2).

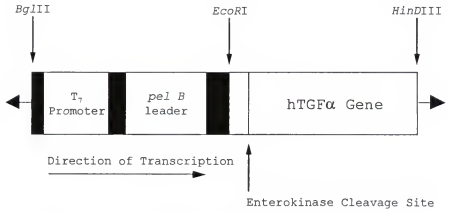



Figure 3-2. pET-22b(+) Expression Vector.

Bacteriophage $T_7$ RNA polymerase is a highly processive RNA polymerase. Essentially, this processivity allows an efficient transcription of a gene. The $T_7$ RNA polymerase also has a highly specific promotor which is unlike any bacterial polymerase promoter, and so the possibility of transcription initiation from a bacterial RNA polymerase is low. As the $T_7$ RNA polymerase originates from the $T_7$ bacteriophage, a bacterial expression plasmid that bears the $T_7$ promotor requires an expression host that carries a copy of the $T_7$ RNA polymerase gene in its chromosomal DNA. The expression hosts containing this gene are often termed $DE_3$ hosts, as they are lysogens of the bacteriophage $DE_3$. The $T_7$ RNA polymerase gene is under the control of a *lacUV5* promoter and the $DE_3$ host contains a *lacI* gene. The bacterial expression hosts used initially were JM109($DE_3$), BL21($DE_3$) and HMS174($DE_3$). BL21($DE_3$) cells had an additional advantage in that they lacked the *lon* and *ompT* proteases, thereby potentially reducing the amount of proteolytic degradation of proteins during purification.(96)

The use of the *lacI* and *lacUV5* promoters essentially allows tighter control of the target gene expression levels. The *lac* repressor acts at both the *lacUV5* promoter in the host chromosome to prevent premature transcription of the $T_7$ RNA polymerase gene and at the $T_7$*lac* promoter in the vector to prohibit transcription of the target gene by any $T_7$ RNA polymerase that is made in the absence of IPTG induction.(71)

Native hTGFα and Mutant hTGFα Gene Assembly

The hTGFα gene was assembled via PCR using a series of deoxynucleotides comprising the 150 bp gene region. The primers were designed for 15 bp overlap between them. Once assembled, the gene was amplified and subsequently cloned into an appropriate PCR cloning vector (either pCR™ or pGEM®-T). The nucleotide sequence was confirmed using dideoxynucleotide sequencing via the Sanger method.(65) Point mutations were made at selected sites by modification of the existing primers to correspond to the desired mutation (Figure 3-3). These mutations were incorporated by the use of overlap extension PCR.(66,67) These mutant hTGFα genes were also subsequently cloned directly into a T overhang vector and their DNA sequences confirmed.

Method Development for hTGFα Protein Expression

The hTGFα gene was ligated into the *EcoRI-HinDIII* sites in the expression vector pET-22b(+) using standard ligation techniques.(97) Shrimp alkaline phosphatase was used to remove the 3' phosphate groups from the pET-22b(+) vector. This ensured that reclosure of the pET-22b(+) plasmid occurred at a lesser extent, thereby increasing the potential for ligation with the hTGFα insert. Initial tests were made to confirm that the hTGFα gene was being expressed, using JM109($DE_3$), BL21($DE_3$), and HMS174($DE_3$) host cells. Very little difference was seen in the hTGFα expression potential of each of these cell lines, thus, we chose to utilize BL21($DE_3$) cells for subsequent experiments. This was mainly due to a lower level of indigenous proteases in

that cell line. Experiments were done with respect to time, temperature and concentration of IPTG (as inducer of protein expression). We found that 37°C, a 3-5 hour induction time and a minimum concentration of 0.1mM IPTG were optimum requirements for protein expression. Later, a concentration of 0.4mM IPTG was adopted to ensure optimal overexpression of the hTGFα gene (Figures 3-4 and 3-5).

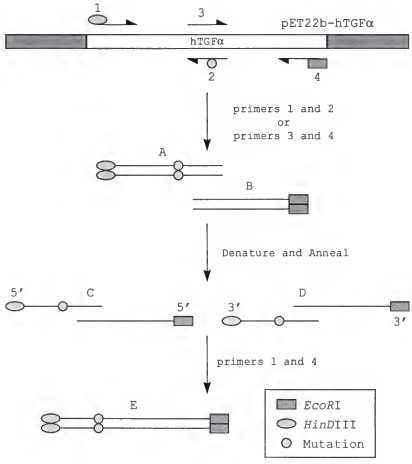



Figure 3-3. Overlap PCR Mutagenesis.

Purification of Expressed *pelB*-hTGFα Protein

Several methods were evaluated to purify *pelB*-hTGFα. Size exclusion chromatography proved to be unacceptable, since the *pelB*-hTGFα eluted either in the void volume or along with higher molecular weight *E. coli* proteins (>20 kDa). The *pelB*-hTGFα protein may be adopting a rod type shape, or aggregating, producing material that elutes differently than typical globular proteins on this size exclusion media. While, we tried several types of media ranging from Sephadex™ G-50 and Sephacryl™ 100, none proved successful in the purification of the recombinant protein.

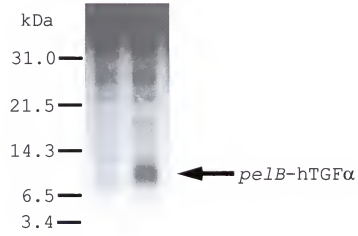



Figure 3-4. SDS-Page Gel of *pelB*-hTGFα.

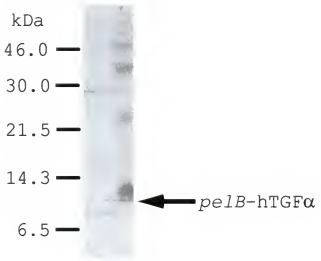



Figure 3-5. Western Immunoblot of *pelB*-hTGFα.

The next method used was ion exchange. An anion exchange column was chosen mainly because the isoelectric point (pI) of *pelB*-hTGFα was 4.5. We employed a diethylaminoethyl (DEAE) column, which is a strong anion exchanger due to its quaternary ammonium groups. The mobile phase used was sodium acetate with a starting pH of 5. *pelB*-hTGFα protein eluted using a gradient from 0-1M NaCl and pH of 4 in a large number of fractions containing various isomers as shown in Figure 3-6. These observations suggested that there was extensive aggregation and misfolding of hTGFα with only a minor proportion of the total hTGFα being active. In order to evaluate the amount of active hTGFα being secreted into the periplasm, we therefore performed radioimmunological and radioreceptor binding assays.

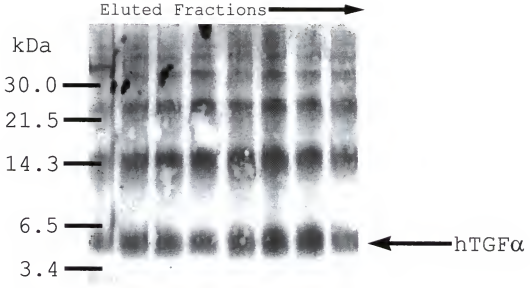



Figure 3-6. Western Immunoblot of DEAE Column Fractions Containing *pelB*-hTGFα.

Enterokinase Cleavage of *pelB*-hTGFα Protein

Enterokinase was chosen as the protease for fusion protein cleavage because it is one of the most specific endoproteases known. It only recognizes the sequence, Asp-Asp-Asp-Asp-Lys, and normally cleaves between the Asp and Lys residues. Enterokinase is somewhat expensive compared to other proteases available, i.e. Trypsin, Factor $X_a$; however, there are negligible amounts of nonspecific cleavage produced when using it.(98,99) Our initial experiments with Biozyme enterokinase showed that cleavage of the *pelB* leader from hTGFα seemed specific, albeit slow, requiring a minimum of 72 hours. We realized later that the specificity of enterokinase was in question.

RIA/RRA Analysis of Expressed Proteins

The analysis of expressed *pelB*-hTGFα protein was performed using both radioimmunometric and radioreceptor assays. A labeled tracer, in this case iodinated hTGFα, was used in a competitive binding assay with unlabeled hTGFα, and a standard curve of commercial hTGFα used to interpolate all assay results. In the immunometric assay, labeled and unlabeled hTGFα compete for binding to a polyclonal antibody specific for full length hTGFα. In the receptor assay, the same species compete for binding to EGF receptor contained in placental homogenate, which consisted of a solution containing fresh placental tissue homogenized with an isotonic salt solution, filtered through fine mesh, and concentrated as described in Chapter 2.

Results were quantified using the logit transformation, and recombinant sample data were interpolated from data generated by the standard curve of commercial hTGFα. (71)

$$\text{logit } b = \ln \frac{b}{100 - b}$$

b= proportion of tracer bound expressed as a percentage of that in the zero standard

Initially, we wanted to see exactly how much active protein was being secreted into the periplasm. The data in Table 3-1 suggested that the amount of fully active *pelB*-hTGFα protein was nominal in terms of the total amount expressed.

Table 3-1. *pelB*-hTGFα Crude Expression Yield.

| | RIA (g/L) | RRA (g/L) | RRA/RIA Ratio |
|---|---|---|---|
| *pelB*-hTGFα Periplasmic Extract | $5.6 \times 10^{-4}$ | $5.6 \times 10^{-5}$ | 0.10 |

There was approximately an order of magnitude difference in the amount of active and nonactive recombinant *pelB*-hTGFα protein in solution. The nonactive portion of recombinant protein probably consisted of multiple forms of incorrectly folded protein. Purification of pure hTGFα must encompass separation from bacterial proteins as well as from isomers of itself. Many different types of size exclusion columns were tested as mentioned previously. The column which produced the best separation, without harsh conditions, was the strong anion exchange column (HQ, Perseptive Biosystems, Cambridge, MA) of the BIOCAD workstation. The packing material on the HQ column contains quaternized polyethyleneimine groups.

The workstation has the capability of simultaneously monitoring pH, conductivity, and absorbances at two wavelengths with flow rates comparable with fast performance liquid chromatography (FPLC). Thus, conditions were optimized using workstation pH and flow rate programs. Final conditions utilized a gradient from 0-2M NaCl in 50mM Tris, 50mM Tris-bis propane, pH 8.5. A reference of pure commercial grade hTGFα was purified under identical conditions. Comparing Figures 3-7 and 3-8, one can see that the reference peak corresponds to the last peak in the elution profile of our crude mixture. Thus, there is only a small percentage of soluble, active, native *pelB*-hTGFα protein.

The major components are *pelB*-hTGFα isomers likely to be in various misfolded states.

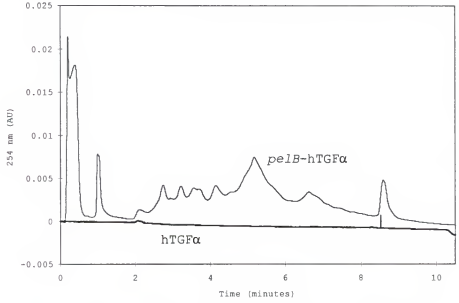



Figure 3-7. DEAE Column Trace of *pelB*-hTGFα and hTGFα.

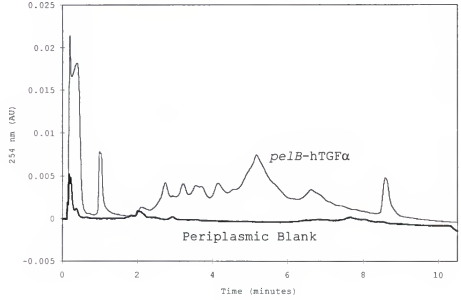



Figure 3-8. DEAE Column Trace of *pelB*-hTGFα and Periplasm.

CHAPTER 4
FINAL EXPRESSION SYSTEM AND PURIFICATION

## Introduction

Building upon the lessons learned in our initial expression system, we now knew that pET-22b(+) was capable of producing *pelB*-hTGFα in large quantities (ca. 0.5 mg *pelB*-hTGFα per liter of culture) but that the amount of active hTGFα was low. Somehow we had to either alter our existing expression system to yield larger amounts of hTGFα in its active form or go to another expression system altogether. Considering the extensive background knowledge we already had on the pET-22b(+) vector, we chose to alter our existing pET-22b(+) expression vector. In the process of finding a more productive and efficient method for expressing soluble, active hTGFα, the pHis*TrxA* plasmid was assembled in our laboratory. A stretch of ten histidine residues (histidine tag) was added to create a quick, facile and reproducible method for purifying fusion protein. Thioredoxin (*TrxA*) was added as a fusion partner to facilitate the production of native hTGFα protein containing the proper disulfide pairings.(100) These two new facets to the pET-22b(+) expression vector and their results are discussed in further detail in the following pages.

## Histidine Tag

A tract of ten histidine residues was placed into the pET-22b(+) vector for ease of purification. There are several advantages to using such an affinity tag.

The frequency of the amino acid histidine occurring in proteins is low. More significantly, large histidine tracts are especially uncommon. Selection for the histidine tag would eliminate most *E. coli* proteins without indigenous histidine.

Selective purification is one of the major benefits of using the histidine tag as it tightly binds divalent cations such as $Cu^{2+}$, $Zn^{2+}$, $Ni^{2+}$, and $Co^{2+}$.(101,102) The use of divalent cations is ruled by strength of affinity: $Cu^{2+}>Ni^{2+}>Zn^{2+}\sim Co^{2+}$. Purification can be tailored to fit several different interaction strengths. We chose nickel ($Ni^{2+}$) since it has a reasonable affinity for the histidine tag, but the affinity is not sufficiently high to require harsh elution methods, thereby preserving disulfide bonds in hTGFα. The ten histidine tract has an affinity orders of magnitude above that of bacterial proteins for divalent cations.(103) Iminodiacetate links in resins are used for divalent charging (Figure 4-2).(104) Some resins, such as Novagen's HisBind™, covalently link the metal to the resin, thus eliminating the need to recharge the column. These types of resins were unacceptable in our experiments as we desired full charging of the column, which is difficult to ensure with covalently bonded metals where metal loss is always a problem.

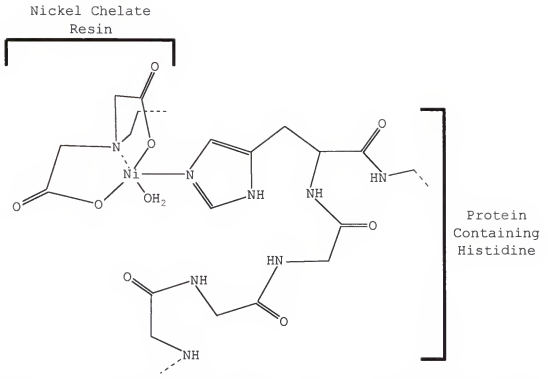



Figure 4-1. Coordination of Nickel with a Histidine Containing Protein.

Lastly, facile purification of histidine tagged proteins enables the processing of large amounts of culture at one time. The use of a physiological pH of 7.9 in all purification buffers allows gentle purification of fusion protein. The protein is loaded onto the column in 20mM Tris, 0.5M NaCl, 5mM imidazole at a pH of 7.9 and eluted in 1M imidazole. Histidine is essentially an imidazole ring, thus, using imidazole in the elution buffer, elutes fusion protein efficiently and reproducibly.

## Thioredoxin

Thioredoxin facilitates the formation of disulfide bonds in some proteins by acting as a catalyst for redox reactions with its two free sulfhydryl groups.(62,64,105) Thioredoxin

(MW 11,700) can exist in either the reduced form (*TrxA*-$(SH)_2$) or oxidized form (*TrxA*-$(S)_2$), where the half cystine residues form an intramolecular disulfide bond.(106) *TrxA*, ubiquitous in bacterial cells, normally catalyzes the reduction of ribonucleotide reductase, an essential enzyme that is involved in DNA synthesis. The S-S bond of oxidized *TrxA* is reduced by NADPH and a specific enzyme called thioredoxin reductase (*TrxB*).(107) LaVallie et al. found that *TrxA* did indeed help the formation of the correct disulfide bonds in insulin growth factor.(100)

His*TrxA*-hTGFα Fusion Protein Expression

There was an increase in the amount of active protein found in crude cytoplasmic extracts, although the use of SOB media was required for the production of the His*TrxA*-hTGFα fusion protein. Little to no protein was produced using lower nutrient media. In addition, long periods of protein expression (ca. 12-24 hours) resulted in cell lysis, thus, protein expression was limited to a maximum of 5 hours. Use of the nickel affinity column affected a quick and specific method for purifying fusion protein from extracts of *E. coli* cytoplasm. Typically, the growth and purification of fusion protein could be achieved in one day.

His*TrxA*-hTGFα Fusion Protein Purification

The detailed analysis of purified fusion protein showed a decrease in receptor binding when compared to that of crude extract. Since all conditions, such as buffer and pH, remain the

same a probable cause was impurities in the $NiSO_4$ that caused the reduction of disulfide bonds resulting in a loss in fusion protein activity. Our experimental results show that the addition of DTT in small amounts (0.4mM) to dialysis buffers destroys the activity of the His*TrxA*-hTGFα fusion protein. When using the purest $NiSO_4$ available (less than 0.1 percent impurities) no difference in the amount of active fusion protein was seen. Experiments conducted with $NiSO_4$ concentrations ranging from 0.5mM to 3.0mM with His*TrxA*-hTGFα protein showed that fusion protein precipitated at a nickel concentration of 1.5mM and above. Native PAGE gels of these same samples showed no distinguishing differences between the precipitated samples and the samples with fusion protein in solution. It is more likely that with prolonged exposure to air, oxygen is oxidizing *TrxA*, as seen by Laurent, allowing hTGFα's disulfides to be irreversibly reduced.(108) This loss of receptor binding ability was sacrificed for the ability to produce and purify more protein in a short amount of time.

An RIA, utilizing polyclonal antibodies, measured the total amount of hTGFα in the solution, regardless of its conformation. An RRA, using EGF receptors from human placental tissue, measured the fraction of hTGFα in solution that binds to the EGF receptor, in a way, measuring the amount of correct folding. The ratio of these two values gives the percentage of correctly folded, biologically active protein in solution, although a definitive analysis of biological activity requires that the recombinant

protein be tested for its ability to stimulate DNA synthesis *in vivo*).

Table 4-1. His*TrxA*-hTGFα Crude Expression Yield.

| | RIA (g/L) | RRA (g/L) | RRA/RIA Ratio |
|---|---|---|---|
| His*TrxA*-hTGFα Cytoplasmic Extract | $4.13 \times 10^{-4}$ | $2.12 \times 10^{-4}$ | 0.51 |

Five times more His*TrxA*-hTGFα protein is able to bind the receptor as seen with the *pelB* expression system, with a ratio of 0.1 as seen in Table 4-1. As there was a large difference in the activity of expressed recombinant hTGFα protein in the two systems, we chose to focus on the production of recombinant hTGFα in the pHis*Trx*ApET22b(+)(pHis*TrxA*) system. While, refolding *pelB*-hTGFα protein was an option, this process was reported to yield only yields 50-60% of correctly folded hTGFα.(86)

*TrxA* has been reported to help the formation of disulfides in some proteins, including interleukin 2 and insulin.(100,105) As thioredoxin is a cytoplasmically expressed protein, usually located at the periphery of the cytoplasmic membrane, we placed ten histidine residues (histidine tag) in front of the *TrxA* gene to serve as an affinity handle in the selective removal of the fusion protein from the other cytoplasmic *E.coli* proteins.(63) The His*TrxA* fragment was subcloned into the *pelB* site in the pET22b(+) vector, effectively replacing the leader protein, directly adjacent to the original multiple cloning site (MCS) in the vector (Figure 4-2). The subsequent addition of the hTGFα gene was done as previously described (see chapter 3). We originally engineered an enterokinase cleavage site in our hTGFα

gene construct, thus a proteolytic cleavage site was not placed into the pHis*TrxA* vector's multiple cloning site.

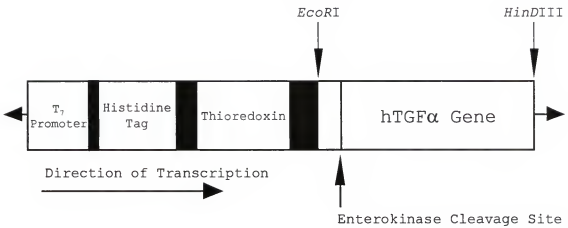



Figure 4-2. pHis*TrxA* Expression Vector.

Expression of the His*TrxA*-hTGFα fusion protein (MW 20,124) was done in the same cell line as with pET-22b(+), BL21($DE_3$) cells. SDS PAGE gel and western immunoblot analysis confirmed the expression of His*TrxA*-hTGFα fusion protein, as seen in Figures 4-3 and 4-4. The growth media was required to be SOB, or any other suitably rich, broth as all other growth media produced little to no fusion protein. Thioredoxin can be expressed in minimal media, thus the media requirements are probably due to the production of the histidine tag.(63) This observation has been reported for several other commercial vectors containing a histidine tag, such as the pET-15b(+) vector containing the His Tag© leader sequence (Novagen, Madison WI).

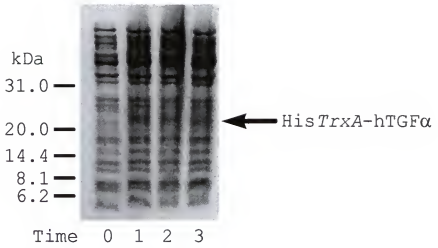



Figure 4-3. SDS-Page Gel Analysis of His*TrxA*-hTGFα Expression.

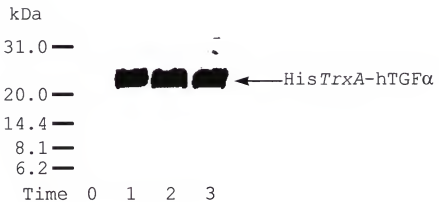



Figure 4-4. Western Immunoblot of His*TrxA*-hTGFα Expression.

Initial purification experiments of HisTrxA-hTGFα were conducted on a Biorad® Econo™ system using the Novagen His Bind™ resin (Novagen, Madison WI). The precipitation of fusion protein through the use of ammonium sulfate increased percentage of correctly folded fusion protein in solution as seen in Table 4-2. This phenomena causes the irreversible precipitation of fusion

protein aggregates, thereby fusion proteins containing the correct disulfide pairings are concentrated through this procedure. Once purification had been optimized, the purification was scaled up to a higher capacity column and system allowing efficient elution of fusion protein as seen in Figure 4-5.

Table 4-2. Purified Wildtype Recombinant His*TrxA*-hTGFα Activity.

| His*TrxA*-hTGFα | RIA (g/L)x$10^{-5}$ | RRA (g/L)x$10^{-5}$ | RRA/RIA Ratio |
|---|---|---|---|
| original purification protocol | 57.0±9.0 | 8.3±0.1 | 0.15±0.02 |
| modified purification protocol | 3.5±0.4 | 3.7±0.6 | 1.1±0.3 |


Figure 4-5. SDS-PAGE Gel of Metal Chelate Affinity Column Fractions Containing His*TrxA*-hTGFα.

This next system used was the BioCAD™ workstation which allows operation under high pressure for rapid protein purification. A standard metal chelate column (MC#089) with iminodiacetate linkages was used for purification. Charging of the column was done with $NiSO_4$ solution and elution was carried

out with imidazole. The process was automated to facilitate rapid production of the His*TrxA*-hTGFα fusion protein. After purification on the nickel column, the sample was processed, cleaved with enterokinase and separated with a Rainin ultramicrofilterfuge. This sample was then analyzed on a $C_{18}$ Vydac column. Pure, fully folded hTGFα eluted at 20 minutes (33% ACN) using a 60 minute linear gradient, from 5-100% ACN at 1 mL/min flow rate, as seen with all previous purifications of hTGFα.(68) Simultaneous injection of commercial hTGFα and recombinant hTGFα, produced from our pHis*TrxA* system, showed a peak coeluting at 33% ACN (20 min, peak 2) as seen in Figures 4-6 and 4-7. Peaks 1,3, and 4 were analyzed by SDS-PAGE gel and western immunoblot and contain no detectable protein.

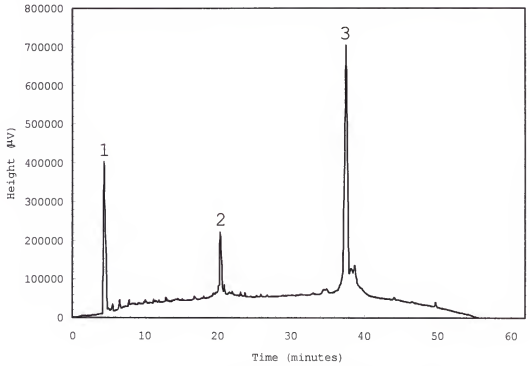



Figure 4-6. HPLC Trace of Recombinant hTGFα.

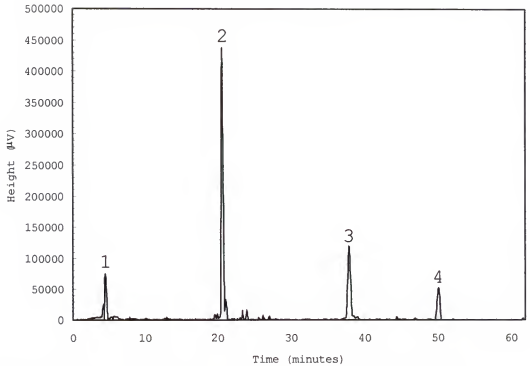



Figure 4-7. HPLC Coinjection of Recombinant hTGFα and Commercial hTGFα

Large amounts of nonspecific proteolytic cleavage were observed when processing the final batches of purified His*TrxA*-hTGFα fusion proteins as seen in Figure 4-8. Nonspecific cleavage is a rare occurrence with enterokinase. However, contaminating proteases from the enzyme's source, in this case calf intestine, could produce this extensive degradation. Since we had previously done small scale (μg amounts) enterokinase digestions upon the His*TrxA*-hTGFα fusion protein, it would have been difficult to realize the large extent of degradation occurring as seen in Figure 4-9. His*TrxA*-hTGFα fusion protein is very stable at the enterokinase reaction temperature of 37°C (Figure 4-10). In addition, no denaturation or aggregation of the fusion protein out of solution occurred as no protein precipitate was seen in the reaction tubes. Unfortunately, due

to large losses suffered from nonspecific proteolytic cleavage by enterokinase, sufficient amounts of recombinant native hTGFα protein were not produced for any further type of analysis.

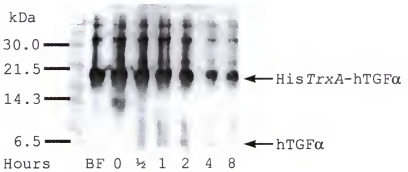



Figure 4-8. Western Immunoblot of Enterokinase Nonspecific Degradation.

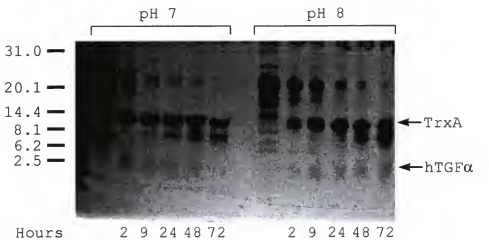



Figure 4-9. SDS PAGE Gel of Enterokinase Cleavage Reactions of His*TrxA*-hTGFα Protein at pH7 and pH8.

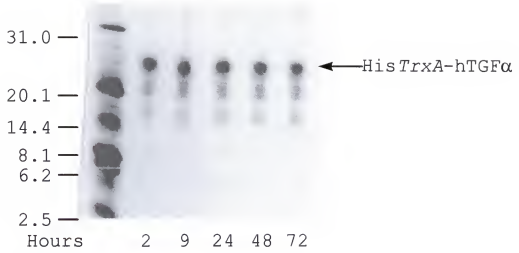



Figure 4-10. SDS-Page Gel of the Stability of His*TrxA*-hTGFα at 37°C.

Instead of expending effort upon optimization of a new source of enterokinase, we decided first to gather all binding and activity data for the ten His*TrxA*-hTGFα fusion proteins, if time permitted a different source of enterokinase would be sought and reaction conditions worked out. Radioreceptor assays conducted upon His*TrxA* protein, hTGFα and His*TrxA* + hTGFα showed no difference in hTGFα concentrations as shown in Table 4-3 and Figure 4-11. Thus, the His*TrxA*-hTGFα fusion protein binding data presented in the next two chapters is a qualitative guide for the binding of recombinant native hTGFα protein and its site specific mutants. P*seudomonas* exotoxin-hTGFα and vaccinia growth factor-hTGFα fusion proteins have also successfully produced biologically active hTGFα fusion proteins.(109-114)

Table 4-3. Contribution to Binding Affinities from His*TrxA* Fusion Protein.

| Sample | $K_D$ (M) |
|---|---|
| His*TrxA* | $(2\pm1)\times10^{-4}$ |
| His*TrxA*+hTGFα | $(1.69\pm0.03)\times10^{-8}$ |
| hTGFα | $(1.66\pm0.03)\times10^{-8}$ |

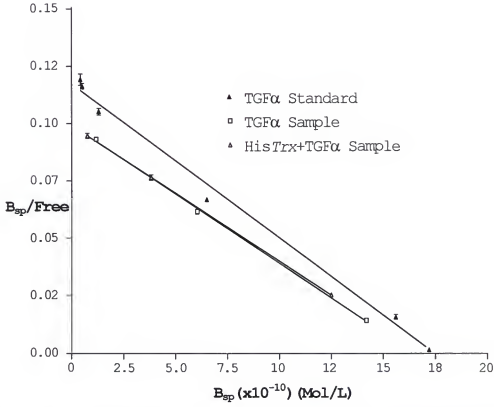



Figure 4-11. Scatchard Plot of His*TrxA*+TGFα and TGFα Compared with the TGFα Standard

CHAPTER 5
VALINE 33 HINGE MUTANTS

## The Hinge Region of hTGFα and Its Relevance

As described previously, hTGFα is composed of 3 major loop domains defined by disulfide bonds. Between the second and third disulfide linkages, there lies a single residue in both hTGFα, residue 33, and hEGF, residue 32 as seen in Table 5-1. Thus, this hinge residue forms the bridge between the N- and C-terminal domains. Hinge residues differ for each EGF growth factor family member (i.e. TGFα, EGF, amphiregulin (AR)) but are conserved within each growth factor type regardless of species (i.e. rat, human, mouse). Referring to Table 5-1, amino acid sequence alignment shows EGF contains an asparagine (Asn) residue, and TGFα contains a valine (Val) residue in the hinge position.

In addition to the conservation of the hinge region, the differences in size and moieties of these hinge residues make them an excellent target for site directed mutagenesis. Since each growth factor type conserves these hinge residues, in addition to their structural impact, the possibility of random chance is low, suggesting an important role for the hinge interactions with the EGF receptor. Our hypothesis, concerning the role of the hinge region, proposes that these residues are major determinants of the N- and C-terminal domain orientation. Change in the size or moiety of the hinge residue may impact the

spatial orientation of the neighboring domains and would be a way that each growth factor type is distinguished by their common receptor, EGF-R. Initial contact with EGF-R may depend upon gross conformational loop orientation which then determines the subsequent biological response.

Table 5-1. Alignment in Valine 33 Region of EGF Family Members.

| **Various members** | |
|---|---|
| Human Amphiregulin | VTC**K**CQQ |
| Human Cripto | -FC**A**CPP |
| Rat Epiregulin | KFC**R**CEV |
| **EGF** | |
| Human EGF | YAC**N**CVV |
| Mouse EGF | YTC**N**CVI |
| Horse EGF | HAC**N**CVV |
| **TGFα** | |
| Human TGFα | PAC**V**CHS |
| Mouse TGFα | PAC**V**CHS |
| Rabbit TGFα | PAC**V**CHS |
| Hamster TGFα | PAC**V**CHS |
| Pig TGFα | PAC**V**CHS |
| Macaque monkey TGFα | PAC**V**CHS |
| Sheep TGFα | PAC**V**CHS |
| Bovine TGFα | PAC**V**CHS |

Extensive mutagenic studies of Asn32 in EGF (Table 5-2) support this idea to a limited extent. In addition, Richter et al., using hTGFα synthetic peptides, showed that both domains were important in EGF receptor recognition.(55) Flexibility in the $\phi$ and $\psi$ angles of the hinge residue correlates to overall conformational flexibility. According to our receptor dimerization model, hinge mutants might have a large impact upon EGF receptor recognition of different growth factor types and subsequent dimerization. However, only two hTGFα Val33 mutants

have been done by previous researchers. Feild et al. changed Val33 to proline (Pro, P).(47) Proline substitution, a cyclic amino acid with fixed $\phi$, $\psi$ angles, resulted in a total loss of receptor binding capability in hTGF$\alpha$. This result is understandable since the restricted torsion angles of proline would allow no fluctuation whatsoever in the domain orientation. Binding to EGF-R is not likely to be a static process, thus, some rotational flexibility is required to establish an interaction. Tam et al. chemically synthesized an alanine (Ala, A) mutant at position 33 of hTGF$\alpha$, and reported this mutation to have a large effect upon receptor binding capability.(58)

Table 5-2. Mutations Done in the Hinge Region of hEGF and hTGF$\alpha$.

| | Residue | Mutation | Binding Affinity* |
|---|---|---|---|
| hEGF | Asn32→ | Lys | 110(115) |
| | | Trp | 100(115) |
| | | Gly | 35(115) |
| | | Asp | 25(115) |
| | | Pro | <0.02(115) |
| | | His | 78(116) |
| | | Val | 46(116) |
| | | Phe | 29(116) |
| | | Asp | 7(116) |
| hTGF$\alpha$ | Val33→ | Pro | «1(47) |
| | | Ala | 20(58) |

*Binding affinity is expressed as percent of the affinity of the corresponding full-length protein.

Care was taken to choose substitutions that would not disrupt formation of the disulfide bonds critical for binding at the EGF receptor. Five substitutions were selected; Val33, was changed to asparagine (Asn, N), lysine (Lys, K), isoleucine (Ile, I), glycine (Gly, G), and methionine (Met, M), whose structures

are depicted in Figure 5-1. Substitutions were chosen either for growth factor specificity, Asn in EGF and Lys in amphiregulin, or structural similarities, as in the case of Ile, or moiety changes, Gly and Met.

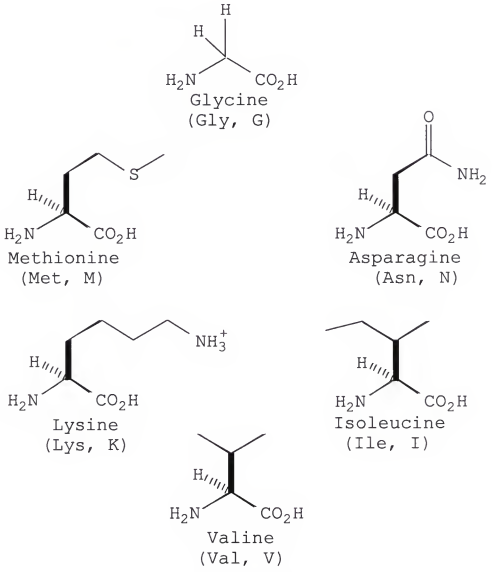



Figure 5-1. Chemical Structures of Valine 33 Substitutions.

Selection of asparagine (Asn, N) for substitution in hTGFα at position 33 not only conserved the hydrophobic character present in wildtype hTGFα but overall size. In addition, as Asn

is exclusively the hinge residue in EGF, we did not anticipate folding problems or major changes in EGF-R binding, allowing this residue to serve as a "weathervane" for evaluating other mutations.

Although lysine (Lys, K) contains a linear four carbon side chain, disulfide pairing should occur normally, as confirmed by Lys in the hinge position in amphiregulin, a fellow member of the EGF family.(3) The V33K mutant should show a slight difference in EGF-R binding since neighboring loop orientation would shift to accommodate the size difference. Lysine's side chain($pK_a$ 10-10.2) is protonated at physiological pH, thus it is hydrophilic in nature. This hydrophilicity is in direct opposition to the normal hydrophobic character of valine. This character difference was expected to result in decreased EGF receptor binding affinity.

Both isoleucine (Ile, I) and valine possess branched carbon side chains differing by only an additional methyl group. This slight size difference should not alter domain orientation to a great extent. The $\phi$, $\psi$ angles in Ile also have similar rotational freedom to those in valine. Hydrophobic character is inherent in Ile, conserving the moiety in the wildtype protein. Receptor binding of V33I was expected to be similar to that of the wildtype protein.

Essentially, glycine (Gly, G) was chosen for flexibility about its main chain torsion angles. Possessing no sidechain, glycine allows us to utilize the V33G mutant as a model for EGF-R binding, a gauge for the behavior of neighboring domain

orientation. Glycine possesses the largest amount of rotational freedom in its $\phi$, $\psi$ angles. It is hard to estimate how large an impact this rotational freedom will have upon EGF receptor binding, although we expect it may destroy receptor binding capability. This flexibility allows the V33G mutant to adopt several different domain conformations, thus lowering the probability that it has the preferred conformation needed binding to the EGF receptor.

Methionine substitution at Val33 was done for other reasons. Absent in wild type hTGFα, methionine can be cleaved by cyanogen bromide (CNBr) producing two distinct regions of hTGFα, the N-terminal domain, consisting of the A and B loops, and the carboxy terminal domain, containing the C-loop. Cyanogen bromide cleavage produces a homoserine lactone at the cleavage site which may be used to attach a synthetically produced domain. This type of cleavage would allow exploration into the binding affinities of the two major hTGFα domains. Specifically, we could see whether either of these regions confers both receptor binding and activation. The production of double mutants with methionine at position 33 may allow quantification of each domains' individual contribution to EGF-R binding and dimerization. This information would foster the production of hTGFα antagonists for use in cancer therapy and possibly agonists for acceleration of wound healing.

Previous studies done on synthesized hTGFα loop structures focused only upon loop variants, not the conformation produced by the native hinge residue.(49,89,117) Defeo-Jones et al. and

Tam et al. synthesized fragments comprising the A-, B-, A-B, and C-loops of hTGFα and found that these fragments exhibited no substantial receptor binding.(49,89,117) In addition, the A- and A-B synthetic fragments contained the omission of N-terminal residues 1-8. Although residues 1-4 could be deleted without consequence in full length hTGFα, deletion of residues 1-7 in hTGFα destroyed receptor binding capability.(49) The C-loop constructs contained no C-terminus tail (residues 44-50), which has also been shown to be critical for receptor binding.(118)

Receptor binding assays were used to ascertain binding capabilities of the His*TrxA*-hTGFα proteins produced in this work. These binding assays utilized a human EGF receptor system. Val33 mutants were initially assayed with the human EGF receptor to ascertain binding capability while receptor activation was quantified by a methyl-$^{3}$H-thymidine incorporation assay in human corneal fibroblast cells. In essence, if the mutated growth factor stimulated cellular growth, DNA synthesis would commence, incorporating methyl-$^{3}$H-thymidine into the growing nucleotide chain as seen in the stimulation curve in Figure 5-2. The stimulation is dependent upon EGF-R dimerization and autophosphorylation signaling event. If a Val33 mutant failed to dimerize the EGF receptor, no DNA synthesis would occur. This would not, however, preclude the Val33 mutant binding to the EGF receptor. In this way, we could determine whether a mutation was involved in both binding and receptor activation.

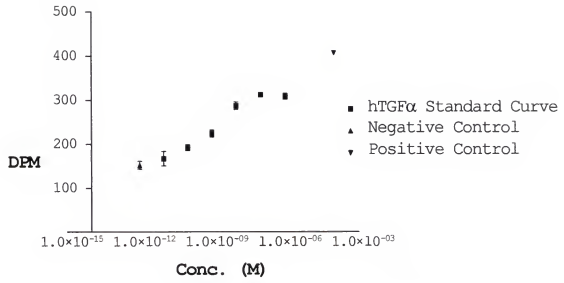



Figure 5-2. Standard Curve of DNA Stimulation by hTGFα

The amount of correctly folded mutant protein was ascertained by RIA/RRA ratios. All RIA hTGFα concentrations calculated for His*TrxA*-hTGFα fusion proteins were within experimental error of RRA concentrations, confirming correct protein folding. Optical density measurements of mutant proteins were also in good agreement with RIA calculated concentrations as shown in Table 5-3. His*TrxA* protein was not recognized by the polyclonal hTGFα antibody used in the RIA as seen by the large discrepancy in $OD_{280}$ and RIA calculated concentrations. In addition, HPLC work, done on a small amount of cleaved wildtype hTGFα protein, confirmed the only protein component in solution was correctly folded hTGFα as shown in Chapter 4.

Table 5-3. Comparison of Calculated Concentrations from $OD_{280}$ Values and RIA Values.

| | $OD_{280}$ conc. (M) | RIA (M) | $OD_{280}$/RIA |
|---|---|---|---|
| wildtype hTGFα | $1.32 \times 10^{-5}$ | $(2.36 \pm 0.90) \times 10^{-5}$ | 0.56 |
| V33N | $7.26 \times 10^{-6}$ | $(1.36 \pm 0.20) \times 10^{-6}$ | 5 |
| V33I | $3.95 \times 10^{-6}$ | $(3.41 \pm 0.60) \times 10^{-6}$ | 1.2 |
| V33K | $4.97 \times 10^{-6}$ | $(2.11 \pm 0.70) \times 10^{-6}$ | 2 |
| V33M | $3.89 \times 10^{-6}$ | $(6.00 \pm 1.00) \times 10^{-6}$ | 0.65 |
| V33G | $1.48 \times 10^{-5}$ | $(3.02 \pm 0.30) \times 10^{-6}$ | 0.49 |
| His*TrxA* | $3.74 \times 10^{-5}$ | $(9.00 \pm 5.00) \times 10^{-11}$ | 41,555 |

Receptor Binding and Activation of the Val33 Mutants

The V33N mutation, shown in Table 5-4 and Figure 5-3, displayed a magnitude difference in binding affinity from wildtype hTGFα. This difference can be seen as negligible since it mimics the difference seen from wildtype fusion protein versus hTGFα standard. As expected, the DNA stimulation capability found with V33N is comparable to that of wildtype (Table 5-5, Figure 5-4 and 5-5). Asn is found in hEGF at position 32 and the binding difference in human receptor systems between hEGF and hTGFα is negligible.(119) Ile, Lys and Met also showed no difference in binding affinity or activation, within experimental error, from that seen with respect to hTGFα standard. His*TrxA* has no binding or stimulation capabilities whatsoever.

Table 5-4. Relative Binding Affinities of Val33 Mutants[†]

| | $K_D$ (M) | $K_D$-wt/$K_D$-mutant |
|---|---|---|
| wildtype hTGFα | $(1.56 \pm 0.03)\times10^{-7}$ | |
| V33N | $(1.08 \pm 0.03)\times10^{-6}$ | 0.146 ± 0.005 |
| V33I | $(1.19 \pm 0.02)\times10^{-7}$ | 1.31 ± 0.03 |
| V33K | $(2.97 \pm 0.06)\times10^{-7}$ | 0.53 ± 0.02 |
| V33M | $(4.4 \pm 0.1)\times10^{-7}$ | 0.36 ± 0.01 |
| V33G | $(7.3 \pm 0.2)\times10^{-6}$ | 0.0214 ± 0.0007 |
| His*TrxA* | $(2 \pm 1)\times10^{-4}$ | 0.0008 ± 0.0004 |
| hTGFα standard | $(1.49 \pm 0.05)\times10^{-8}$ | 10.5* ± 0.4 |

*$K_D$-wt/$K_D$-hTGFα

[†]All proteins were His*TrxA* fusions except in the case of the hTGFα standard.

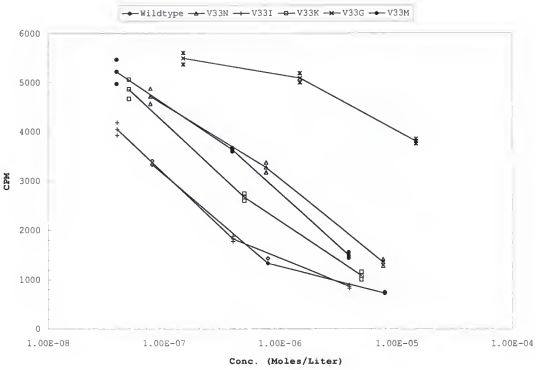



Figure 5-3. Relative Binding Affinity Graph of Val33 Mutants.

Table 5-5. Mitogenic Stimulation Capability of Valine 33 Mutants[†§]

| Trial 1 | DPM | DPM-wt/DPM-mutant |
|---|---|---|
| wildtype hTGFα | 12492 ± 1591 | |
| V33N | 10560 ± 916 | 1.2 ± 0.2 |
| V33M | 11176 ± 1723 | 1.1 ± 0.2 |
| V33G | 9020 ± 294 | 1.4 ± 0.2 |
| hTGFα standard | 5542 ± 851 | 2.3* ± 0.5 |
| **Trial 2** | **DPM** | **DPM-wt/DPM-mutant** |
| wildtype hTGFα | 4083 ± 310 | |
| V33I | 3706 ± 239 | 1.1 ± 0.1 |
| V33K | 3769 ± 94 | 1.08 ± 0.09 |
| hTGFα standard | 3097 ± 97 | 1.3* ± 0.1 |

*DPM-wt/DPM-hTGFα

[†]All proteins were His*TrxA* fusions except in the case of the hTGFα standard.

[§]Two separate trials were done to accommodate all of the samples. Each trial is only compared with its internal standards.

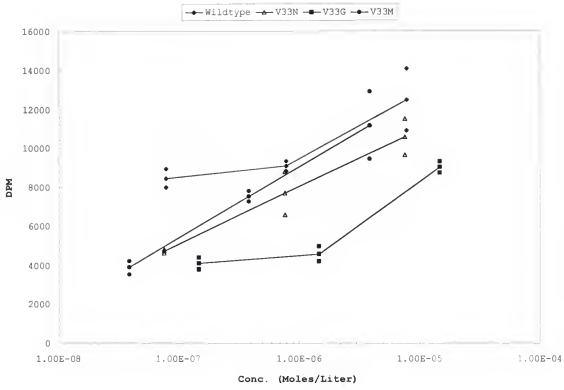



Figure 5-4. Mitogenic Stimulation of Val33 Mutants (Trial 1).

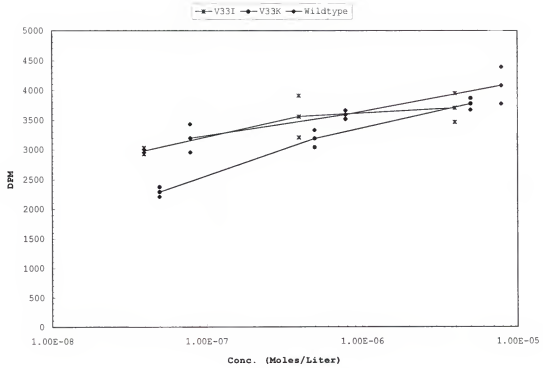



Figure 5-5. Mitogenic Stimulation of Val33 Mutants (Trial 2).

The V33G mutant ($K_d$ 7.3 ± 0.2 μM) displayed almost two orders of magnitude less than the wildtype's receptor binding affinity ($K_d$ 0.158 ± 0.003 μM). The most plausible explanation lies in the fact that glycine has more rotational flexibility in its $\phi$, $\psi$ angles allowing more movement about the hinge residue.

As a result, multiple domain arrangements would limit the ability of the V33G mutant to adopt the proper conformation for receptor binding. Since solution structures of the V33G mutant are currently unavailable, this explanation remains to be established definitively. Yet, the receptor activation capability of V33G was within experimental error of that seen

with wildtype. This suggests that the flexibility seen in V33G may have limited its ability to adopt the proper conformation needed for binding to the EGF receptor. Yet, once that conformation was adopted, binding and subsequent activation were similar to wildtype. This result lends support for our multidomain binding hypothesis of hTGFα to the EGF receptor.

Although binding differences were small in magnitude for the human EGF-R, an alternate EGF-R receptor source might exhibit altered receptor binding interactions. As the chicken EGF receptor shows a 100 fold difference in binding between hTGFα and hEGF, this receptor system has potential use in characterizing the Val33 hTGFα mutants. For example, chimeras constructed from hTGFα/hEGF show significant binding affinity differences in the chicken receptor system.(119)

CHAPTER 6
PHENYLALANINE 15 MUTATIONS

## Biological Significance of Phe15 in hTGFα

hTGFα contains three major loops consisting of antiparallel β pleated strands. Each loop region may have an important role in EGF-R binding. Again, as seen in Figures 1-4 through 1-6, the hydrophobicity of hTGFα is mainly grouped into certain specific areas. The B and C loops form a hydrophobic face upon which hTGFα may bind to the EGF receptor. Mutations done by Lazar et al. showed a large correlation between reduction in EGF-R binding and mutational changes in these loops.(45,46) The hydrophobic effect, essentially governed by an increase in solvent entropy, may be the main determinant of these regions interacting with EGF-R.

The A loop which contains amphiphilic and hydrophilic residues is located on what can be considered the "back face" of hTGFα, while the B loop and portions of the C loop comprise the "front face" of the molecule. In our model, residues located on the front hydrophobic face initiates binding of hTGFα to the EGF receptor as the first important event in eliciting a biological response. The back face may therefore control any the dimerization event involving a neighboring EGF receptor. The EGF-R is thought to control dimerization, autophosphorylation of

tyrosine residues in the cytosolic domain of EGF-R and subsequent downstream signaling events leading to cell mitosis.

Different residues were introduced into the A loop residue 15 (Phe15, F). We mutated the highly hydrophobic phenylalanine residue 15 (Phe15) to tyrosine, leucine, asparagine and aspartic acid. hEGF contains a tyrosine at the cognate position Tyr13 as seen in Table 6-1. The F15Y mutant was done to see if the dimerization event would be modified as indicated by methyl-$^{3}$H-thymidine incorporation. Thus, the binding was anticipated to be similar to that in human EGF-R assays, but the methyl-$^{3}$H-thymidine assay might show a difference in the amount of total DNA produced.

Leucine is structurally similar to phenylalanine when one considers the bottom half of the phenylalanine group. This F15L mutation might not show a change in binding if the interaction needed is purely due to hydrophobicity and not residue size or aromaticity. The two charged residues, Asn and Asp, were chosen for their small size and charged groups. These would probe whether hydrophobicity or size is a factor in binding and dimerization events. The proximity of a cysteine also had to be considered as Phe15 is directly next to Cys16, which is involved in disulfide bond with Cys32. Changing the type of residue too drastically could inhibit proper disulfide formation. Thus, proline substitution was not considered as it has rotational rigidity and may deter correct disulfide pairing.

Table 6-1. Alignment in Phenylalanine 15 Region of EGF Family Members.

| | |
|---|---|
| **Various members** | |
| Human Amphiregulin | FQN**F**CIH |
| Human Cripto | NRT**C**CLN |
| Rat Epiregulin | MDG**Y**CLH |
| **EGF** | |
| Human EGF | HDG**Y**CLH |
| Mouse EGF | YDG**Y**CLN |
| Horse EGF | YDG**Y**CLH |
| **TGFα** | |
| Human TGFα | HTQ**F**CFH |
| Mouse TGFα | HTQ**Y**CFH |
| Rabbit TGFα | HTQ**F**CFH |
| Hamster TGFα | HTQ**F**CFH |
| Pig TGFα | HSQ**F**CFH |
| Macaque monkey TGFα | HTQ**F**CFH |
| Sheep TGFα | HTQ**F**CFH |
| Bovine TGFα | HSQ**F**CFH |

Table 6-2. Previous Mutations at Position 13 of hEGF and Position 15 of hTGFα.

| | **Residue** | **Mutation** | **Binding Affinity*** |
|---|---|---|---|
| hEGF | Tyr13→ | Phe | 97(120) |
| | | Leu | 78(120) |
| | | Leu | 202(121) |
| | | Ile | 22(120) |
| | | Val | 20(120) |
| | | His | 16(120) |
| | | His | 8(122) |
| | | Arg | 6(120) |
| | | Ala | 3(120) |
| | | Gly | 0.3(120) |
| hTGFα | Phe15→ | Ala | 1(49) |
| | | Ala | 136(58) |

*Binding affinity is expressed as percent of the affinity of the corresponding full-length protein.

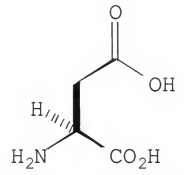
Very few mutations have been done at position Phe15 in TGFα (Table 6-2). Defeo-Jones et al. created a β-gal fusion TGFα protein with a F15G mutation and this mutant exhibited only 1% of

the wildtype's receptor binding ability.(49) This may be due to flexibility issues, as was discussed for V33G in Chapter 5, or it may be due to the lack of a critical interaction not being formed. Mutants at position Tyr13 in EGF have been investigated by several groups. Most notably, Tadaki et al., did a Tyr13Phe mutant which showed 97% of the wildtype's receptor binding ability.(120) Thus, we would expect a similar type of result in our Phe15Tyr mutant. Two positively charged mutations, Y13R and Y13H, were done at residue 13 in EGF. Arginine is fairly basic with a $pK_a$ of 12-13, while histidine is only mildly basic with a $pK_a$ of 6-7, close to neutral. Both of these mutations at EGF residue Y13 drastically reduced the receptor binding ability to 6 and 16% of that observed for wildtype TGFα activity respectively. This leads us to believe a similar type of result for our F15D and F15H mutations. No other charged residue substitutions at EGF residue Y13 have been reported.

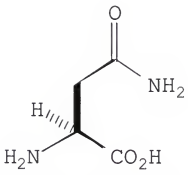
We wished to perform substitutions at phenylalanine 15 that would test our EGF receptor binding and dimerization theory, results are in Table 6-3. The five engineered substitutions were tyrosine, leucine, aspartic acid, asparagine and histidine as seen in Figure 6-1.

Table 6-3. Comparison of Calculated Concentrations from $OD_{280}$ Values and RIA Values.

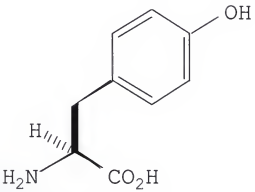
| | $OD_{280}$ conc. (M) | RIA (M) | $OD_{280}$/RIA |
|---|---|---|---|
| wildtype | $1.32 \times 10^{-5}$ | $(2.36 \pm 0.90) \times 10^{-5}$ | 0.56 |
| F15Y | $1.30 \times 10^{-6}$ | $(1.21 \pm 0.70) \times 10^{-6}$ | 1.1 |
| F15D | $2.83 \times 10^{-5}$ | $(2.00 \pm 1.00) \times 10^{-5}$ | 1.4 |
| F15N | $1.17 \times 10^{-5}$ | $(8.00 \pm 1.00) \times 10^{-6}$ | 1.5 |
| F15L | $1.78 \times 10^{-5}$ | $(5.00 \pm 2.00) \times 10^{-5}$ | 0.36 |
| His*TrxA* | $3.74 \times 10^{-5}$ | $(9.00 \pm 5.00) \times 10^{-11}$ | 41,555 |



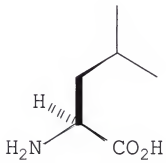

Aspartic Acid
(Asp, D)



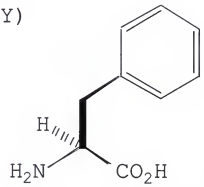

Asparagine
(Asn, N)




Tyrosine
(Tyr, Y)




Leucine
(Leu, L)




Phenylalanine
(Phe, F)

Figure 6-1. Chemical Structures of Phe15 Substitutions.

The histidine (His, H) mutation was the only unsuccessfully cloned gene with all attempts yielding several additional point

mutations in addition to the F15H mutation despite extensive efforts. In several cases, we produced clones that had no relationship in common to the original hTGFα template. We can only surmise that the F15H mutation is somehow unfavorable and must be cloned using techniques other than overlap PCR.

Tyrosine (Tyr, Y) was chosen based on the cognate residue in epidermal growth factor. In addition, tyrosine is structurally similar to phenylalanine with only an additional hydroxyl group at the para position in benzene. Tyrosine still possesses hydrophobic character despite the hydrogen bonding capability of its hydroxyl group, and so this mutation conserves the original moiety at this position, we expected no major difference in EGF receptor binding or activation.

The second mutation, leucine (Leu, L), mimics the branched carbon chain of phenylalanine. Leucine should also pose no structural problem with respect to hTGFα folding and disulfide formation Its substitution mainly probes whether a benzene ring or merely hydrophobic character is required at position 15. We expected to see an effect in receptor binding and activation if an aromatic residue is required.

The aspartic acid (Asp, D) mutation at position 15 was selected to investigate whether the negative charge could be accommodated during EGF receptor binding and activation. This fact may also play a role in receptor binding if a specific sized binding pocket governs the interaction between residue 15 and EGF-R. Aspartic acid ($pK_a$ 4.4-4.6) is charged at physiological

pH, thus, unfavorable electrostatic interactions with EGF-R may reduce its binding affinity and subsequent kinase activity.

Lastly, asparagine (Asn, N), a mostly hydrophobic residue, was placed at position 15 to test if a benzene ring was required. In addition, it was unclear whether a branched carbon chain was necessary for receptor interaction or did any linear carbon chain suffice. Again, asparagine is smaller in size with respect to phenylalanine, so no abnormalities in terms of hTGFα folding or disulfide bond formation should be seen.

Receptor Binding Affinity of Phe15 Mutants

Table 6-4 and Figure 6-2 show the binding affinities gathered for the Phe15 mutants. As predicted, the F15Y mutant showed a similar $K_D$ value to that of wildtype as well as comparable DNA stimulation ability. There was a slight reduction in binding affinity which might be explained by the presence of misfolded protein. The F15L mutant showed an even larger decrease in $K_D$ value, however mitogenic signaling was not affected. This may point towards a specificity for aromatic residues at position 15 for initial interaction with EGF-R. This specificity was seen in hEGF.

The most striking reduction in binding came from the F15D and F15N mutants. These mutants showed less than 1% and 2% of the wildtype's binding affinity but no corresponding loss in mitogenic signaling (Table 6-5 and Figure 6-3). This is again, likely due to be a requirement for aromaticity. These results suggest that there is a hydrophobic binding pocket in EGF-R.

Table 6-4. Relative Binding Affinities of Phe15 Mutants[†]

| | $K_D$ (M) | $K_D$-wt/$K_D$-mutant |
|---|---|---|
| wildtype hTGFα | $(1.21 \pm 0.02)\times10^{-7}$ | |
| F15Y | $(1.89 \pm 0.04)\times10^{-7}$ | 0.64 ± 0.02 |
| F15D | $(2 \pm 1)\times10^{-5}$ | 0.01 ± 0.03 |
| F15N | $(5.3 \pm 0.5)\times10^{-6}$ | 0.023 ± 0.002 |
| F15L | $(9.8 \pm 0.8)\times10^{-7}$ | 0.12 ± 0.01 |
| His*TrxA* | $(2 \pm 1)\times10^{-4}$ | 0.0006 ± 0.0003 |
| hTGFα standard | $(1.83 \pm 0.03)\times10^{-8}$ | 6.6* ± 0.2 |

*$K_D$-wt/$K_D$-hTGFα
[†]All proteins were His*TrxA* fusions except in the case of the hTGFα standard.

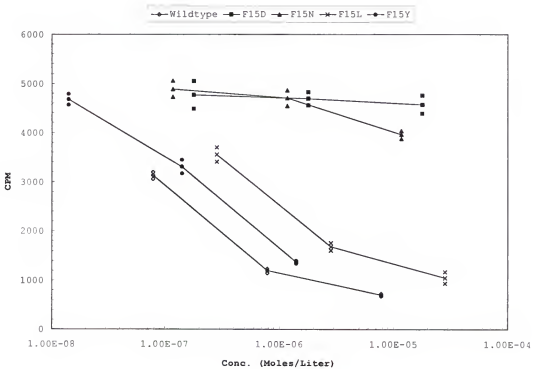



Figure 6-2. Relative Binding Affinities of Phe15 Mutants.

Table 6-5. Mitogenic Stimulation Capabilities of Phe15 Mutants[†§]

| | DPM | DPM-wt/DPM-mutant |
|---|---|---|
| wildtype hTGFα | 12492 ± 1591 | |
| F15D | 6280 ± 1241 | 1.9 ± 0.5 |
| F15N | 7124 ± 763 | 1.8 ± 0.3 |
| F15L | 10355 ± 1361 | 1.2 ± 0.2 |
| F15Y | 12621 ± 392 | 1.0 ± 0.1 |
| hTGFα standard | 5542 ± 851 | 2.3* ± 0.5 |

*DPM-wt/DPM-hTGFα

†All proteins were His*TrxA* fusions except in the case of the hTGFα standard.

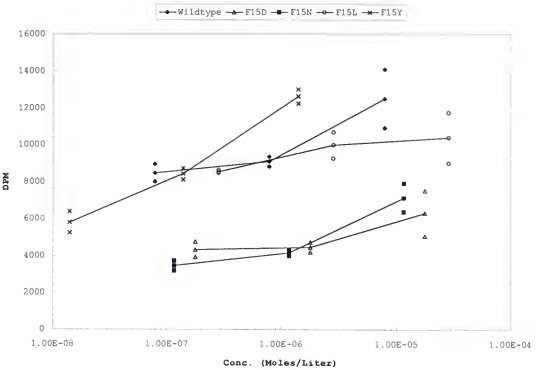



Figure 6-3. Mitogenic Stimulation of Phe15 Mutants.

# CHAPTER 7
# CONCLUSIONS AND FUTURE WORK

## Conclusions

Our research upon human transforming growth factor alpha had three specific goals. The initial aim of this research was to develop a protein expression system for the production of correctly folded hTGFα. The pHis*TrxA* protein expression system we developed enabled us to produce large amounts (over 1 mg of fusion protein per liter of culture) of correctly folded recombinant *TrxA*-hTGFα fusion protein. The use of both thioredoxin and a histidine tag has proven both innovative and amenable to facile manipulation. The attainment of this goal allowed us to express nine hTGFα mutants and determine their relative binding affinities and biological activities. Furthermore, the facile purification system produced milligram amounts of *TrxA*-hTGFα fusion protein in a short amount of time.

Our initial target was the hinge residue of hTGFα, Val33, which lies directly between two critical disulfide bonds. Thus, it is logical to hypothesize that this residue may have a large impact upon overall domain orientation, and thus, receptor recognition. Sequence homology alignments show that the valine residue is conserved at position 33 for all proteins in the TGFα family. From this knowledge, we hypothesized that residue 33 may be a major determinant for initial EGR receptor binding and have

tested this concept through a series of mutations at valine 33. The valine 33 mutants of hTGFα have shown interesting trends with respect to their receptor binding affinities. The most notable one, V33G, lends support for the hypothesis concerning initial receptor recognition. The flexibility of the V33G mutant clearly reduces its EGF-R receptor binding potency, yet, once bound V33G is capable of eliciting the same biological response. Using the dimerization model, these results suggest that the V33G mutant has a higher efficacy in the stimulation of EGF-R dimerization than the wildtype.

Our final aim was to determine the importance of Phe15 in EGF receptor binding and activation. This highly conserved residue is located in what we believe is the putative receptor binding pocket of hTGFα. Phe15 may also have a limited impact upon the EGF receptor dimerization process. This type of hydrophobic core interaction is similar to that proposed for the interactions seen with human growth hormone and its receptor.(57)

The phenylalanine 15 mutants of hTGFα also show that residue 15 is critical for receptor binding. The large reductions in binding affinities in the F15D and F15N mutants may point toward a unique binding pocket that can not accommodate ionized or polar residues. Our findings suggest this pocket may only be able to accommodate hydrophobic residues. As seen with the V33 mutants, the relative efficacy of the F15 mutants is the same as seen with wildtype TGFα, again suggesting a role for F15 in EGF receptor recognition.

Thus, our specific aims have been achieved with new insights into the structural role of residues 15 and 33 in hTGFα interactions with the epidermal growth factor receptor which supports our multidomain receptor binding model.

## Future Work

There are several areas that can be expanded and improved in this presented research. These areas include enterokinase source, in-depth structural analysis and determination of dimerization response of the V33G, F15D, and F15N mutants and an alternative receptor assay system. In addition, the dimerization process can be explored through site directed mutagenesis of Ser11 in hTGFα's A-loop.

### Enterokinase Cleavage Reaction

The enterokinase digestion reaction was less than optimal in our system. Thus, to utilize our expression system more effectively, a new source of enterokinase is recommended. A likely source is Enterokinase Max® from Invitrogen, which is a recombinant enzyme, expressed in *Pichia pastoris*, containing only the catalytic subunit of bovine enterokinase. Thus, contamination from nonspecific bovine proteases, seen in our experiments, should be negligible with this recombinant source. Studies by Mikhailova et al. also found that trace amounts of proteinases leads to degradation of chimeric or fusion proteins.(123) Reaction conditions would need to be reoptimized for this new enzyme source. Utilization of a suitable

enterokinase source would realize the full potential of the novel pHis*TrxA* expression system.

NMR Structural Analysis of hTGFα Mutants

The interesting binding affinities afforded by the V33G, F15D and F15N mutants would be enhanced with corroborating structural data from NMR experiments. Our expression system is capable of producing milligram quantities of protein needed for NMR, yet, specific isotopic label enrichment in our proteins is unfeasible as we are unable to utilize the minimal type growth medium in this expression system.(124,125) This would not preclude the use of a different expression system, such as a yeast expression system, merely for the production of isotopically labeled protein.

NMR structural determination of the hTGFα/EGF-R complex could be done using methods similar to that of McInnes et al.(126) Chemical exchange of hTGFα on and off the EGF-R as well as the dynamics of hTGFα when bound to the EGF receptor would be possible using NMR relaxation measurements. Several techniques used for characterization of the bound ligand's environment include transferred NOE and multinuclear multidimensional NMR, such as $^{13}C$ and $^{15}N$ relaxation measurements. The use of $^{1}H$ NMR relaxation of methyl residues in hTGFα, as done by McInnes et al., is quite effective in the determination of hTGFα/EGF-R interactions.(126)

There are several reasons to utilize the measurement of methyl relaxation behavior. A primary reason is the prevalence

of methyl groups, with 14 out of 50 amino acids in hTGFα containing a total of 22 methyl groups. Methyl groups are well distributed throughout the backbone of hTGFα, thus, no regions of hTGFα are ignored by this type of relaxation measurement. NMR resonance assignments, through 2D and 3D NMR, have been extensively done, thus every methyl group has been completely assigned and well resolved. In addition, methyl groups have an intensity three times that of a single proton resonance with simple coupling patterns of uniquely identifiable doublets.

Using 1D NMR spectra, line broadening due to binding of hTGFα to the EGF-R is very apparent. Residues experiencing the greatest amount of line broadening often show a corresponding reduction in their mobility, suggesting some type of close association with the EGF receptor. Thus, it is possible to discriminate regions within hTGFα that participate only nominally in EGF-R binding from regions that are more intimately associated with EGF-R.

Using this type of NMR experiment, investigation of the impact of the V33G mutant upon hTGFα domain orientation may enable refinement of the model for hTGFα/EGF receptor interaction. Differences in the line broadening of methyl resonances within the domains should be seen when comparing the V33G and wildtype hTGFα NMR spectra. Line broadening within the B-loop and C-terminal residues would be expected as these regions should have intimate contact with the EGF receptor according to the dimerization model. Yet, due to the flexibility of the V33G mutant, line broadening may take effect slowly, and hopefully on

a time scale slow enough to detect via NMR, due to the slow change of the mutant protein to a conformation which can bind to the EGF receptor. Such line broadening differences would corroborate our hypothesis of the flexibility of main chain torsion angles in the V33G mutant. These NMR experiments may be able to estimate the on/off rates of V33G, F15D and F15N when binding to the EGF-R which would shed light upon the unusual difference in efficacy and potency of these mutants. The on/off rates of each mutant should correlate with the $K_d$ values calculated in this research.

These structural findings would only indicate interactions within the initial hTGFα/EGF-R complex and would not be able to fully distinguish any type of interactions within a dimerization complex. This type of dimerization complex would have to be ascertained through other means, such as phosphorylation experiments.

Dimerization Event Analysis

The model of EGF-R dimerization through intermolecular interaction, suggested by Schlessinger, is a major point in our description of the unusual interactions seen with several of our mutants. Our main claim to the difference in potency of these point mutants is the manner in which they establish EGF-R dimerization. Although the V33G, F15D and F15N mutants displayed notable decreases in receptor binding efficacy, their potency was equivalent to that seen in wildtype hTGFα. This suggests that their ability to establish EGF-R dimerization is somehow enhanced

through their mutation, whether by conformational changes within hTGFα or another type of interaction. The use of mitogenesis experiments is incapable of measuring the extent to which this dimerization event is altered as it measures only the end point in stimulation. What is needed is an experiment that would determine whether or not dimerization is seen with these mutants, and if so, to what extent it is altered or enhanced to establish their similar potencies.

An experiment most amenable to resolving this type of discrepancy is an assay which establishes whether signaling via autophosphorylation is actually occurring and its quantification. This type of experiment has been extensively used by Yarden and Schlessinger to great utility.(30) They have shown that EGF-R phosphorylation is dependent upon the receptor concentration squared which is consistent with an intermolecular mechanism for tyrosine kinase activation. Dilution of EGF-R concentration thus, would have an effect upon the dimerization event, making it harder for dimers to form effecting subsequent EGF receptor autophosphorylation.

Thus, if V33G, F15D or F15N is important in receptor dimerization and is equipotent to wildtype hTGFα, using a low concentration of EGF-R should show a diminished response for both proteins. Yet, these mutants may be more effective than wildtype hTGFα in eliciting a biological response, suggesting that they could exhibit a higher incidence of dimerization regardless of low receptor concentration, in essence merely a shift in the dose-response curve from that of wildtype. Thus, this type of

behavior should occur with a steady rate of phosphorylation when EGF-R concentration is decreased. Nonetheless, the dimerization event would be harder to achieve due to low receptor concentration and proximity.

Chemical cross linking of EGF-R can also be used to establish if the biological response is due to the dimerization process. Crosslinking of the EGF receptors would enable the measurement of EGF receptor's basal response without ligand. Thus, quantitation of the amount of receptor phosphorylation present with mutant would be possible. The same basal response would be seen if phosphorylation is merely dependent upon receptor proximity and not interaction with ligand. Crosslinking experiments with free EGF receptors as well as receptors with ligands already bound would help elucidate the role of the mutants in dimerization. We would expect to see no difference in phosphorylation when comparing the mutants and the wildtype hTGFα since the autophosphorylation event is due mainly to proximity of the EGF receptor.

A final type of experiment dealing with phosphorylation would be the immobilization of the EGF-R. Immobilization enables the receptor autophosphorylation to be studied. As with the dilution experiment, immobilization of the EGF-R would in a sense dilute the number of receptors within a given area to a finite amount. In this manner, we could corroborate evidence from cross linking experiments. The mutants should show no significant effect since the EGF receptor would not be allowed to readily

attain a clustered state. Thus, a decreased rate of phosphorylation would be seen.

Future hTGFα Mutants

Another avenue to explore is site directed mutagenesis of residues on the "rear" face of hTGFα. These "rear" residues are mainly comprised of A-loop members and some C-loop residues, although there are exceptions as in the case of Phe15 which lies on the periphery of the boundary between the two faces of hTGFα. Using the aforementioned receptor binding hypothesis, mutation of amino acid Ser11 would be a true test of whether the A-loop residues are critical for initiating the EGF receptor dimerization event. Ser11 is conserved in both hTGFα and EGF, suggesting that this residue is important in some manner for interaction with the EGF receptor. However, since no distinction is made between EGF and hTGFα at this position, this fact suggests that Ser11 is involved in interactions after the initial receptor binding/recognition event is over. Ser11 is probably integral in the receptor dimerization and autophosphorylation event. This would make sense with our model of the hinge residue controlling the hTGFα conformation and the Ser11 initiating contact with another EGF receptor causing autophosphorylation to occur. This model would suppose that the binding event is the single most critical event in recognition of the ligand with the phosphorylation event being a byproduct of this binding with no difference in activation. However, this type of interaction could also be explained via the model proposed earlier. There are

several conserved residues within the A-loop of hTGFα, thus, this would suggest a type of interaction needed for dimerization, most of these residues are capable of hydrogen bonding or electrostatic interactions. Thus, mutation of Ser11 should have a significant effect upon whether the dimerization event occurs at all. Four mutations of Ser11 are suggest, asparagine, aspartic acid, threonine, and alanine.

Serine, with methanol as its side chain, is capable of hydrogen bonding interactions. Changing this amino acid to an electrostatic residue such as Asp would change its moiety to a charged residue. This would disrupt any type of hydrogen bond interaction of Ser11, likely creating a charge-charge repulsion between S11D and the contact residue(s) on EGF receptor. We would except to see a corresponding decrease in potency of this mutant, the efficacy however should remain the same since, according to our model, the binding site would not be affected.

Next, the alanine mutant was chosen since it is a good indicator of what placing a neutral residue at Ser11 would do for its binding and activation. We would expect, as with the S11D mutant, a decrease in dimerization and hence potency, however, this decrease should not be as drastic as that seen with the S11D mutant as we are not substituting a residue that could create potential problems in terms of unfavorable interactions with contact residues on the EGF receptor. Again, the efficacy or receptor binding ability should be similar to that seen with the wildtype.

The next mutant, Ser11Asn was chosen to substitute a residue that would cause an interaction different from that already seen at that position. Asn is polar and capable of hydrogen bonding whereas Asp is charged. Thus, Asn should not cause any type of unfavorable electrostatic interactions. We would expect the interaction of S11N to cause a reduction in potency but not as large a potency reduction as should be seen with the S11D mutant. Again, receptor binding capability of S11N should be on par with that of the wildtype.

Lastly, the Ser11Thr mutant was chosen for its similarity to the original residue serine. Threonine differs from serine only contains its hydroxyl on the beta carbon in a three carbon sidechain whereas serine has its hydroxyl group on a beta carbon in a two carbon sidechain. This difference should not inhibit the hydrogen bonding ability of Thr, however, the presence of a neighboring methyl group may structurally inhibit the ability of Thr to establish contact with the EGF-R if the interaction site is in a deep cleft of some sort. However, judging from serine's smaller side chain, this interaction cleft could not be too large, thus, this type of effect may be unseen.

This series of mutations would serve to test our model of hTGFα interaction and activation of the EGF receptor.

Use of Different Receptor Sources

In addition to NMR, a new type of receptor system would enable us to see subtle differences in receptor binding. Two experiments that would enable the use of different receptor

systems would be the use of the chicken EGF receptor and the use of the erbB family of receptors.

The chicken fibroblast system could be used to exploit its 100-fold affinity difference for EGF and hTGFα.(119,127,128) Thus, mutants of hTGFα could be gauged for their affinity switch in terms of changes in chicken binding specificity. The human EGF receptor shows no such binding difference. Most studies have studied merely the human EGF receptor, however this large difference in binding affinity, present in the chicken EGF receptor, can be used to further characterize how the V33 and F15 hTGFα mutants affect receptor binding and activation. Since the chicken and human EGR receptor share 75% homology in their extracellular domain, 97% homology in the tyrosine kinase domain, and 65% homology within the C-terminal residues, information gathered using this alternate receptor should prove applicable in the human system. The V33 and F15 series of hTGFα mutants should show even larger differences in efficacy, than apparent in the human system, when assayed in the chicken receptor system.

The use of the erbB family of receptors to measure the amount of transmodulation of signal as done by Riese et al. for wildtype hTGFα, EGF, amphiregulin, and heparin binding EGF.(129) In their study, Riese et al. studied the stimulation of the erbB family of receptors towards each of the aforementioned ligands using cell lines expressing the four erbB receptor types, including the EGF receptor (erbB-1), neu (erbB-2/HER2), erbB-3 (HER3), and erbB-4 (HER4) receptors, in single and pairwise combinations. The rate of stimulation was measured using

tyrosine phosphorylation capabilities., Transmodulation refers to a process where EGF family peptides are known to induce phosphorylation and signaling of erbB family receptors that, when expressed alone, are not phosphorylated. These researchers found that the aforementioned ligands were capable of stimulating tyrosine phosphorylation of neu, erbB-4, and in some cases, erbB-3, in an EGF-R dependent manner and are capable of activating the EGF-R in the absence of additional erbB family receptors. Consequently, activation of a single erbB family receptor can transmodulate any of the other three erbB family receptors. Cells may be able to control their growth through the activation of different sets of erbB receptors in this manner.

The use of our V33 and F15 hTGFα mutants in such a mixed receptor assay would allow a closer investigation of whether several specific residues are capable of initiating receptor binding and subsequent dimerization and signaling. Since Riese established that there is a small amount of transmodulation between the different erbB receptors towards different EGF-like ligands, it may be possible to enhance this transmodulation with the use of these mutants as ligands. Through mutation of the V33 and F15 residues, the point mutants may be capable of enhancing the amount of transmodulation seen between the different erbB receptors.

The V33 series of mutants would be ideal candidates for initial study of this transmodulation effect since these mutants have shown that conformation does have an effect upon efficacy. These mutants' capability of similar levels of potency regardless

of efficacy level may be an indicator of transmodulation capability with receptors unable to interact with wildtype hTGFα. The same may be true for the F15 series of hTGFα mutants. However, as this experiment deals with several variables that have been untested with the introduction of hTGFα mutants, the exact response would be hard to gauge.

These future experiments are merely an attempt to bring together the information learned via this dissertation and project what useful information could be obtained through further exploration and study of this unique protein, transforming growth factor alpha.

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## BIOGRAPHICAL SKETCH

Susan Brita Rasmussen was born on August 23, 1969, in Fort Sill, Oklahoma. She spent the most of her life in the bustling metropolis of Copperas Cove, Texas. In May of 1987, she graduated third in her class from Copperas Cove High School. With the several scholarships she earned she was able to attend undergraduate studies at Texas Woman's University in Denton, Texas. While attending courses, she studied chemistry under the tutelage of Dr. Lyman Caswell and Dr. James E. Johnson. She graduated cum laude with a bachelor degree in chemistry from TWU in May, 1991. In the fall of 1991, Susan entered the University of Florida to obtain a degree in Chemistry. She entered the newly formed biochemistry division under the guidance of Dr. Nigel G.J. Richards. For the past six years, she has studied the protein hTGF$\alpha$ and its underlying structure-function relationships. In May 1997, she will be conferred the degree of Doctor of Philosophy and be the first student to graduate from the Biochemistry Division in the Chemistry Department.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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